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Evaluation of Heterologous Promoter Function in the Eastern Oyster (Crassostrea Virginica).

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EVALUATION OF HETEROLOGOUS PROMOTER FUNCTION IN THE EASTERN OYSTER (*CRASSOSTREA VIRGINICA*)

A Dissertation

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ABSTRACT

The goal of this work was to evaluate the feasibility of using antimicrobial peptide gene transfer to enhance oyster immunity. The specific objectives were to: 1) compare the efficiency of collagenase, pronase, and trypsin to dissociate atrium, ventricle and mantle into individual cells for the initiation of primary cell culture; 2) develop cryopreservation conditions to preserve the dissociated cells; 3) establish a serum-free transfection system; 4) evaluate inducible promoter function using the established serum-free transfection system; 5) evaluate the effects of the transfer of antimicrobial peptide genes on oyster immunity.

Pronase was found to be the most effective enzyme for dissociating the atrium, ventricle, and mantle. Thereafter, pronase was used to dissociate atrium and ventricle tissues for development of cryopreservation conditions to preserve cells for culture.

Dimethyl sulfoxide, glycerol, and propylene glycol were evaluated for their effectiveness as cryoprotectants. Freezing rates and thawing temperatures were compared to minimize the damage. The optimal conditions were 10% glycerol, freezing at a medium rate (vials containing cells were packed into a 21 x 19 x 7 cm and 0.7 cm thick polystyrene foam box and with 80 g of cotton followed by equilibration at 25° C for 20 min, –80° C for 16 h, and –196° C for storage) and thawing at 45° C for atrial cells, and 10% glycerol, freezing at a medium rate and thawing at 25° C for ventricle cells.

A serum-free transfection system for ventricle cells was established. A heat inducible promoter was evaluated in the system. The optimal heat shock conditions for the heat shock protein 70 promoter from snail, *Biomphalaria glabrata*, was 40° C for 1h followed by recovery at 25° C for 12 h. The induction of the cecropin B promoter from

moth, *Hyalophora cecropia*, was evaluated in ventricle cells using lipopolysaccharide and IL-1-alpha. *In vivo* transfer of an antimicrobial peptide gene was conducted.

Although not significantly different, oysters receiving the antimicrobial genes controlled by the cecropin B promoter tended to have lower bacteria tissue loading than the controls. The results of this work implied that antimicrobial peptide gene transfer to enhance oyster immunity is feasible.

CHAPTER 1 INTRODUCTION

Goal and Objectives

Disease is a major hindrance in the development of the oyster industry. The hypothesis of this work was that the transfer of an antimicrobial peptide gene to the oyster could result in enhanced immunity to resolve the disease problem. The goal of this work was to evaluate the feasibility of using antimicrobial peptide gene transfer to enhance oyster immunity. The specific objectives were to: 1) compare the efficiency of collagenase, pronase, and trypsin to dissociate atrium, ventricle and mantle tissues into individual cells for the initiation of primary cell culture; 2) develop cryopreservation conditions to preserve the dissociated cells; 3) establish a transfection system for the dissociated ventricle cells which were cultured in serum-free medium; 4) evaluate inducible promoter function using the established cell transfection system; 5) evaluate the effects of the transfer of antimicrobial peptide genes on oyster immunity.

Production of Oysters

Oyster aquaculture is a commercially important industry in the United States, generating a national dockside value of over $100 million in 1995 (MacKenzie 1996a). In 1997 oysters harvested from Louisiana contributed $50 million of the national value (Avery 1998). The eastern oyster, *Crassostrea virginica*, comprises 80% of the United States oyster harvest (Lorio and Malone 1994) and is a native species found along the Atlantic coast of North America from the Gulf of St. Lawrence to the Gulf of Mexico where water temperatures range from 0° C to 30° C (Galtsoff 1964). The eastern oyster is the major oyster species cultured in the United States (Wendell and Malone 1994).

Oysters can reverse their gonad function from male to female, with gonads maturing as males first, then changing to female the following year (Coe 1943). Mature oysters for spawning can be collected from the wild during spawning months (June to September in Louisiana) or year-round from laboratory conditioned (24° C) oysters. Production of seed oysters either collected from the wild, or artificially spawned and fertilized in the hatchery is as follows: male and female ripe oysters are induced to spawn in separate tanks either by shifting temperature from 21° C, to 28° C for 45 min or by chemical stimulation such as 1 μM serotonin (5-hydroxytryptamine creatininesulfate complex) (Gibbons and Castagna 1984). Alternatively, gametes can be stripped from gonads for fertilization at a sperm to egg ratio of 5,000:1 (Castagna and Kraeuter 1981). At 24° C, fertilized eggs develop into embryos in 4 to 8 h, to the trochophore stage in 12 h, and to straight-hinge larvae in 24 h. Further development to eyed larvae (pediveligers) occurs between 12 to 21 days. Then they metamorphose (change body shape) within 72 h, attach themselves to a substrate and form a shell. The later stage is called spat, which can be seeded on sea grounds leased by farmers from the government. The spat will reach maturity in 1 to 2 years (Galtsoff 1964).

Oyster Disease

Disease is one of the major obstacles for the oyster industry. At least 45 oyster diseases, for which the causative agents are either identified or unidentified, have been reported. The main diseases associated with the eastern oyster and Pacific oysters, *C. gigas*, are summarized in Table 1-1 (Bower et al. 1994). Causative agents include viruses, bacteria, and protozoa. The most notorious disease threatening the culture of the eastern oyster is Dermo disease, which is caused by the protozoan parasite, *Perkinsus*

Table 1-1. Major diseases associated with eastern oysters *Crassostrea virginica* (CV) and pacific oysters *C. gigas* (CG).

Disease	Infected species*	Causative reagent	Invaded tissue
Virus			
Oyster velar virus disease	CG	Icosahedral DNA virus	Velar epithelium
Hemocytic infectious virus disease	CG	Icosahedral DNA virus	Hemocytes
Viral gametocytic hypertrophy	CV	Papillomavirus-like papovavirus	Gametes and gametogenic epithelium
Herpes-type virus disease	CG / CV	Herpes-type virus	Undefined
Bacteria			
Rickettsia-like and Chlamydia-like organisms of oysters	CG / CV	Rickettsiales	Epithelial cells of gill and digestive gland
Nocardiosis	CG	Nocardia sp.	Mantle, gill, adductor muscles and heart
Hinge ligament disease of juvenile oyster	CG / CV	Cytophaga-like (gliding) bacteria	Hinge ligament
Larval and juvenile vibriosis	CG / CV	*Vibrio* sp.	Necrosis of soft tissue
Protozoa			
Dermo disease	CG / CV	*Perkinsus marinus*	Inhibition of growth, development of gonads, disruption of connective tissue
Oyster egg diseae	CG / CV	Protozoan	Cytoplasm of mature ova
Seaside organism (SSO)	CV	*Haplosporidium costale*	Connective tissue of digestive gland, gonads and mantle
Multinucleate sphere X (MSX)	CV	*Haplosporidium nelsoni*	Digestive epithelia
Aber disease	CG	*Marteilia refringens*	Tissue necrosis, cessation of growth
Denman Island Disease	CV	*Mikrocytos mackini*	Vesicular tissue necrosis
Gill trichodina	CG	*Tricodina* spp.	Erosion of gill
Larval mycosis	CV	*Sirolpidium zoophthorum*	Disintegration of the soft tissues
Shell-burrowing sponges	CG / CV	*Cliona* spp.	Penetration of shell and formation of holes
Oyster trematode diseases	CG / CV	*Gymnophalloides tokiensis*, *Bucephalus* spp.	Retardation of growth
Unknown			
Malpeque Disease	CV	Unknown	Mantle recession and oedema

marinus, and results in an annual mortality of 50% in Gulf Coast oysters (Craig et al. 1989). *P. marinus* not only inhibits the growth of oysters but also the development of gonads, and subsequently leads to a decrease in reproductive capacity (Kennedy and Breisch 1981, Paynter and Burreson 1991).

The methods of diagnosis, treatment, and prevention of oyster diseasess are few. The molecular diagnosis *of Haplosporodium nelsoni* (Stokes and Burreson 1995), and *P. marinus* (Yarnall et al. 1997) uses the polymerase chain reaction, and the immunodiagnosis of protozoa (Mialhe et al. 1988, Boulo et al. 1989) and *Vibrio* spp. (Noël et al. 1996) uses polyclonal and monoclonal antibodies. However, most other diseases, such as viral diseases, require diagnostic methods that still rely on morphological description and traditional histology. The lack of cell lines and well-established cell culture conditions for the isolation of pure virus further hinders studies of viral diseases at the molecular level. The control of oyster diseases primarily relies on management practices. Although treatment for bacterial diseases has been evaluated, it is not practical for application in open-sea beds, and the induction of antibiotic resistant strains could result. Therefore, control strategies include quarantine, destruction of virus infected oysters, and culturing oysters at low salinity and in low temperature areas to reduce the prevalence of *P. marinus* or harvesting oysters early before the high mortality of *P. marinus* infection occurs (Mackenzie 1996b).

Oyster Immunity

Oysters do not have adaptive immunity, characterized by specificity and memory of previous exposure to pathogens, but they do possess innate non-specific immunity. The first line of innate oyster defense against the invasion of pathogens is the hard shell

and mucus covering the soft body. The second line of defense is the collaboration of cellular and humoral immunity. Immunologists currently use the term "cellular immunity" to refer to the specificity of immune responses of T lymphocytes and the term "humoral immunity" to refer to the specificity of antibody responses of B lymphocytes. However, oyster researchers and those who study more primitive animals such as invertebrates still use the term "cellular immunity" to refer to non-specific immune responses of phagocytic cells and the term "humoral immunity" to refer to any effector molecules in the circulation of hemolymph. In this dissertation, the terms cellular immunity and humoral immunity are used in the broad sense of the terms used for oysters and invertebrates.

The limited knowledge of oyster immunity makes its study particularly challenging. It requires an examination of the immune response of closely related bivalves and other mollusks and even further, references to unrelated invertebrates and vertebrates. This makes the establishment of universally accepted terminology more critical and hopefully it will be addressed soon.

Cellular Immunity

Hemocytes: Oysters have an open circulatory system. The hemolymph (blood) containing hemocytes circulates over tissues after passing out of arteries with open ends and returns to the heart from a sinus. However, the hemopoietic tissues of bivalves are not known (Cheng 1981, Hine 1999). The classification of oyster hemocytes relies on morphology and is divided into two types: granulocytes and agranulocytes (Cheng 1981). Both hemocyte types are active in phagocytosis of small particles and encapsulation of

particles that are too large to be engulfed by a single cell, but granulocytes are the most phagocytic cell type (Foley and Cheng 1975).

Phagocytosis: The most primitive mechanism of cell defense is phagocytosis. The attraction between oyster hemocytes and pathogens relies mainly on chemotatactic molecules released from pathogens (Cheng and Howland 1979). Then, attachment between pathogens and hemocytes can be enhanced (opsonized) by Ca^{++} (Renwrantz and Stahmer 1983) and lectins, which reside in hemolymph or on the surface of hemocytes (Vasta et al. 1982). Thereafter, the pathogens are enclosed as membrane-bound vacuoles, phagosomes, and fuse with lysosomes containing various enzymes and antimicrobial factors (Cheng and Rodrick 1975).

Effector Molecules of the Hemocytes

Reactive oxygen intermediates (ROI): Once the pathogen is taken into the lysosome, its elimination relies on the effector molecules of the hemocyte. Phagocytosis in molluscs is an oxygen consuming reaction. Bursts of respiratory activity during phagocytosis generate reactive oxygen intermediates (ROI) including superoxide anions (O^{2-}), hydroxyl radicals ($^{\cdot}OH$), singlet oxygen ($^{\cdot}O_2$), and hydrogen peroxide (H_2O_2) (Adema et al. 1991). However, pathogens may not stimulate hemocytes to produce ROI. For example, the ROI are not produced when live meronts, a multicellular stage in life cycle of *P. marinus*, are phagocytized by eastern oyster hemocytes, but heat-killed *P. marinus* meronts stimulate the production of ROI (La Peyre et al. 1992, 1995, Volety and Chu 1995). Failure of ROI generation after hemocyte phagocytosis of *P. marinus* may contribute to the spread of Dermo disease.

Nitric oxide: Bivalve hemocytes also produce nitric oxide (NO) for generating peroxynitrite anions ($ONOO^-$), which are highly toxic in the presence of superoxide anions (Beckman and Koppenol 1996). The NO can be found in the hemocytes of blue mussels, *M. edulis*, and freshwater snails, *Viviparus ater*, (Conte and Ottaviani 1995) but these compounds have not yet been identified in oysters.

Lysosomal enzymes: Other effector molecules in bivalve lysosome include lysozyme, alkaline phosphatases, non-specific esterases, indoxyl esterase, beta-glucuronidase, lipase, aminopeptidase, lysozyme and beta-hexosaminidase. Few studies of these enzymes have been conducted with the exception of lysozyme (Cheng and Rodrick 1975). Lysozyme is an enzyme that can digest the peptidoglycan layer of bacterial cell walls. The lysozyme of oysters is similar to egg white lysozyme, which is a 15 kilodalton (KD) basic protein with the ability to lyse bacteria (Rodrick and Cheng 1974). The lysozyme concentration of oysters is subject to seasonal variation and inversely related to salinity (Chu and La Peyre 1993).

Humoral Immunity

Lysosomal enzymes in hemolymph: circulation factors also play roles in pathogen elimination. The release of lysosomal enzymes such as acid phosphatase into hemolymph is observed upon bacterial challenge in marine bivalves (Cheng and Buter 1979) but their regulation has not been identified.

Agglutinin: Agglutinins are non-immunoglobulin proteins containing 20 KD of identical subunits that can agglutinate particles such as bacteria (*Vibrio cholerae*), by binding specifically with sugar moieties on the particles (Acton et al. 1969). Agglutinins can exist either free floating in the oyster hemolymph or associated with oyster

hemocytes (Vasta et al. 1982). The main functions of agglutinin are 1) immobilization and inactivation of pathogens to facilitate subsequent encapsulation, or extracellular killing by other humoral factors, 2) as a mediator (opsonin) to facilitate phagocytosis, and 3) as self and non-self molecular recognition (Renwrantz and Stahmer 1983, Mullainadhan and Renwrantz 1986). The agglutinin concentrations in the hemolymph of oysters challenged with *P. marinus* and *H. nelsoni*, and unchallenged oysters show no difference implying that it was not induced (Chinatla et al. 1994).

Hemolysin: Circulation defense factors that can lyse red blood cells are referred to as hemolysins. They were not found in oysters but are found in hemolymph and hemocytes of quahogs, *Mercenaria mercenaria*, and blue mussels. Hemolysin is inducible and requires calcium ions for function (Hardy et al. 1976, Feng and Barja 1986). Its role in bivalve humoral immunity is not known.

Antimicrobial peptide: Antimicrobial peptides are found in invertebrates and vertebrates. For molluscs, including sea hare, *Dolabella auricularia*, (Kisugi et al. 1992), Mediterranean mussel, *M. galloprovincialis*, (Hubert et al. 1996a), blue mussel (Charlet et al. 1996), and Pacific oyster (Hubert et al. 1996b), antimicrobial peptides are found in hemocytes or hemolymph to protect from pathogen invasion. For example, defensin-1 of Mediterranean mussel (MGD 1) is a 4 KD peptide containing 60 amino acid residues. The granules of hemocytes are the major sites where MGD 1 is found and released to the hemolymph in response to bacterial challenge (Mitta et al. 1999).

Cytokine-like factor: Important effector molecules found in hemolymph of molluscs are cytokine-like factors (Table 1-2) (Clatworthy 1998) that can mediate and regulate the immune response (Ottaviani and Franceschi 1997). These factors were

Table 1-2. Cytokine-like molecules found in molluscs.

Molluscs	Species	Cytokine-like molecules
Mussel	*Aplysia californica*	Interlukin-1-(IL-1)-beta, Tumor necrosis factor (TNF)-alpha
Snail	*Planorbarius corneus*	IL-1-alpha, IL-1-beta, IL-2, IL-6, TNF-alpha
Snail	*Viviparus ater*	IL-1-alpha, IL-1-beta, IL-2, IL-6, TNF-alpha
Snail	*Biomphalaria glabrata*	IL-1-beta, TNF-alpha
Mussel	*Mytilus edulis*	IL-1-alpha, IL-1-beta, IL-2, IL-6, TNF-alpha

produced from blue mussel hemocytes with antigenic stimuli such as LPS (Hughes et al. 1991). Although there is no direct evidence that bivalve cytokines participate in disease resistance, the biological activities of cytokines cross function among species. For example, human IL-1 alpha and TNF-alpha induce similar morphological changes (increase cell perimeter) in blue mussel granular hemocytes as seen in human granulocytes (Hughes et al. 1990) and mammalian cytokines (IL-1 alpha, IL-1 beta, IL-2, IL-6, and TNF-alpha) affect the cell mobility, phagocytosis, and induction of nitric oxide synthase of hemocytes in snails (*Planorbarius corneus* and *Viviparus ater*)(Ottaviani and Franchini 1995). Conversely, IL-1 like-molecules are found in starfish, *Asterias forbesi*, (Beck and Habicht 1991).

Enhanced Oyster Immunity by Transfer of Disease Resistance Genes

Although the eastern oyster possesses innate immunity as described above, these effector molecules obviously are not enough to protect oysters from the invasion of certain fatal pathogens such as *P. marinus*. Because the eradication of pathogens is not possible, and quarantine and vaccines are not feasible to control oyster diseases, introduction of non-indigenous genes and genetic selection of disease resistance strains are two major strategies being evaluated to resolve oyster disease problems. Eastern oyster culture could be replaced by culture of the pacific oyster that is resistant to *P. marinus* (Barber and Mann 1994), but other pathogens such as herpes-like virus (Hine et

al. 1992, Le Deuff et al. 1996) are emerging to endanger hatchery-reared pacific oysters (Table 1-1). In addition, induction of exotic species may cause ecological impacts and lead to the loss of indigenous species. Therefore, it is not the best resolution for the problem of oyster diseases (Andrew 1980). Strains resistant to MSX have been successfully developed by genetic selection, which is a passive, slow, and a less controllable technique, the MSX resistance strains are still susceptible to other diseases such as Dermo, and the specific disease resistance gene for MSX has not been identified (Gaffney et al. 1996). In contrast to traditional genetic selection, genetic modification of oysters by transferring disease resistance genes is an active, fast, and more controllable strategy to establish disease resistant strains.

Oyster Cell Culture

Cell culture has been long recognized as an important tool for cellular and molecular studies. For example, the ability to isolate viruses in cell culture can enhance disease diagnosis, while the pure virus obtained from cell culture can facilitate research on further characterization of the virus.

Vertebrate cell culture and cell lines are well established, but marine invertebrate cell culture lags far behind (Rinkevich 1999). Although attempts to culture oyster cells were successful with heart and mantle cells as early as 1960, further progress of oyster cell culture and establishment of cell lines has been limited (Table 1-3)(Chen and Wen 1999). The major causes of failure are attributed to the lack of systematic approaches and inappropriately adopting vertebrate cell culture conditions (Bayne 1998). The efficiency of dissociation enzymes used to dissociate tissue has not been systematically compared.

Table 1-3. Reports of culture conditions for oyster cells (after Chen and Wen 1999).

Species	Year	Tissues	Basal Medium
Ostrea edulis	1960	Heart and mantle	Seawater
Crassostrea virginica	1964	Mantle	Blue crab plasma
C. virginica	1966	Heart	M199 and seawater
C. virginica	1966	Heart	Scherer's medium and balanced salt solution
C. virginica	1979	Heart	2 x M199
C. virginica	1979	Hemocyte	Minimum essential medium (MEM)
C. virginica	1981	Heart, gonad, mantle, palp. Larvae, and neoplastic hemocyte	NCTC-135, RPMI 1640, or L-15
C. virginica	1985	Mantle and heart	MEM
C. virginica	1987	Mantle and embryo	Ham's F-12
C. virginica	1989	Embryo	Ham's F-10
C. virginica	1992	Visceral ganglion	2 x L-15
C. gigas	1993	Heart	2 x L-15
O. edalis	1995	Heart	L-15 and seawater

Therefore, Chapter 2 addresses the systematic comparison of tissue dissociation efficiency of enzymes for culture in a medium optimized for oyster cells.

Cryopreservation of vertebrate cells is an essential part of cell culture to reduce the cost and accidental loss of cells, but no cryopreservation conditions have been developed for oyster cells. Therefore, Chapter 3 addresses the development of the first cryopreservation conditions for oyster somatic cells.

Gene Transfer Methods

After the establishment of successful culture and cryopreservation procedures, a gene delivery method must be determined and optimized to deliver candidate antimicrobial peptide genes into cells for evaluation. Methods used to deliver genes can be categorized into biological, physical, and chemical methods.

One of the most often used biological methods is the use of viruses as a vector to deliver target genes into host cells. The virus genome is modified to reduce the generation of toxic proteins, the transformation of cells into malignant cells, and the induction of host immune responses to the transformed cells. The advantage of using viruses as a vector to deliver genes is, that target genes carried in a viral genome can be carried to the nucleus for expression. However, without receptor modification, virus host specificity may limit this use to certain animals.

Retroviruses and adenoviruses are often used to deliver genes into cells. The advantages of using a retrovirus, which is an RNA virus, are: 1) stable integration into host genome, 2) insertion capacity of a gene up to 10 kb, and 3) broad host range. The disadvantages are: 1) the requirement of proliferating host cells for virus to integrate, 2) the difficulty of producing highly infective viruses, 3) possible mutation of viruses

thereby causing disease (Cournoyer and Caskey 1993, Gilboa 1990). Retroviruses have been used in the production of transgenic aquatic animals, including dwarf surfclam, *Mulinia lateralis*, (Lu et al. 1996), zebrafish, *Danio rerio*, (Lin et al. 1994), and medaka, *Oryzias latipes*, (Lu et al. 1997). The major advantage of using an adenovirus, which is a double-strand DNA virus, is that proliferating and non-proliferating cells can be infected. The disadvantages are: 1) non-integration and, 2) causing host humoral and cellular immune responses to virus and transfected tissues (Yei et al. 1994, Yang et al. 1995).

Sperm is a biological method to deliver target genes into oocytes. The uptake of foreign DNA was first found with rabbit sperm in 1971 (Brackett et al. 1971). It has been used to deliver target genes into aquatic animal oocytes including sea urchins, *Paracentrotus lividus*, (Arezzo 1989), Japanese abalone, *Haliotis divorsicolor*, (Tsai et al. 1997), and several fish species (Muller et al. 1992).

Physical methods to deliver genes into cells include microinjection, electroporation, and particle bombardment (gene gun). Microinjection, which is directly injecting the gene through a needle into the nucleus, is time consuming, but a transgene can be precisely located into cells for expression. It has been widely using in producing transgenic mammalian animals such as mice (Landel 1991) and aquatic animals including fish (Inoue 1992) and pacific oyster (Cadoret et al. 1997). Electroporation uses an intensive electric field to make transient pores in the cell membrane allowing macromolecules to enter or exit the cells (Benz and Zimmermann 1981). The advantages of electroporation are simplicity, and the large numbers of cells that can be processed at one time. The disadvantages are the requirement of large amounts of DNA and that it is difficult to perform on internal tissues. It has been widely applied to cells including

yeast, fungi, protozoa, algae (Shigekawa and Dower 1988, Weaver 1995), and medaka (Inoue et al. 1990), and red abalone, *Haliotis rufescens*, (Powers et al. 1995). The gene gun uses high velocity DNA-coated microparticles driven by high-pressure gas to deliver DNA into cells (Yang et al. 1990). Genes can be delivered into most cell types but the coating of particles (usually gold) is expensive. Transgenic fish (Zelenin et al. 1991), and brine shrimp (*Artemia*) (Gendreau et al. 1995) were produced using a gene gun to deliver genes into cells.

Chemical methods basically rely on the positive charge (cationic) of a chemical reagent forming a DNA-reagent complex to aid DNA delivery. Cationic lipid-based liposome reagents are most often used. The reagents possess polar and non-polar groups of molecules that can self-assemble to form liposomes in a solvent. The liposome has a structure similar to the lipid bilayer structure of cell membranes to aid gene delivery (Nicolau and Cudd 1989).

Cationic lipid-based transfection reagents are biodegradable, highly efficient gene delivery vehicles with low toxicity. However, not every cell type can be transfected. Screening proper transfection reagents and optimizing the transfection conditions are required for each specific cell type.

In summary, each gene delivery method has its advantages and disadvantages. For the development of an oyster *in vitro* gene transfer system, a lipid-based reagent was chosen to deliver genes into oyster cells due to the high efficiency of transfection and ease of preparation. Therefore, the first part of Chapter 4, addresses the optimization of transfection conditions using a lipid-based transfection reagent.

Promoter to Control the Expression of a Disease Resistance Genes

Although all the effector molecule genes of immunity can be considered for transfer to enhance oyster disease resistance, antimicrobial peptide genes may have the most potential. Because antimicrobial peptides are nonspecific effector molecules, the antimicrobial peptide genes must be expressed in an inducible way to prevent over expression and damage to host cells.

Gene expression can be regulated at the level of gene transcription, RNA processing, transportation of mRNA from nucleus to cytoplasm, mRNA translation, mRNA stability, and post-translation (Darnell 1982). However, gene regulation is mainly controlled at the transcriptional level by the promoter. The promoter is a segment of DNA to which RNA polymerase and general transcription factors bind, to initiate transcription. Two consensus sequences are found in the promoter region of most genes. The TATA box (consensus sequence of 5'-TATAAAA-3') centers around position – 25, i.e. 25 nucleotides upstream from the transcription start nucleotide (+1). The CAAT box (consensus sequence of 5'-GGCCAATCT-3') centers around position +90 (Breathnach and Chambon 1981). Specific gene regulatory proteins (transcription factors) bind to specific sequences around the promoter region to regulate (inhibit, induce or enhance) gene transcription (Atchison et al. 1988). The presence of specific DNA sequences around a promoter region implies that the gene can be regulated by the transcription factors that bind to those sequences. In addition, the transcription efficiency of a gene can be facilitated by enhancers, which are DNA sequences that can function regardless of orientation and distance from a promoter (Ptashne and Gann 1990). Moreover, the non-

coding DNA sequence (intron) intervening between DNA coding sequences (exons) affects promoter activity. For example, the human beta-globin gene fails to produce stable transcription in erythrocytes unless the intron is included in the transcription unit (Collis et al. 1990).

Because gene expression is mainly controlled at the transcriptional level by the promoter, the selection of an inducible promoter is critical to drive the expression of an antimicrobial gene. Therefore, the second part of Chapter 4 addresses the evaluation of inducible promoters to control gene expression *in vitro*. Chapter 5 addresses the feasibility of gene transfer to enhance oyster disease resistance. Finally, the overall results are summarized in Chapter 6. The chapters were prepared in journal format and modified to dissertation style according to *CBE Style Manual* (Council of Biology Editors 1994).

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CHAPTER 2 COMPARISON OF EASTERN OYSTER TISSUE DISSOCIATION USING COLLAGENASE, PRONASE AND TRYPSIN

Introduction

The eastern oyster, *Crassostrea virginica*, of the Gulf of Mexico and eastern coast of the United States, has been plagued by the widely epidemic and fatal disease Dermo, caused by an intracellular parasite *Perkinsus marinus* (Andrews 1988, Craig et al. 1989). In hatcheries, mortality associated with a virus was found in larvae of the pacific oyster *Crassostrea giga* (Hine et al. 1992), and unknown agents have caused high mortality in spat of the eastern oyster (Small 1997). Although final resolution of these diseases may rely on *in vivo* genetic selection, gene transfer, chemotherapeutants, or modified culture practices, an *in vitro* cell culture system would facilitate and expedite disease research at the cellular and molecular level. Because no transformed cell lines are available for oysters, primary cultures have been used in the study of cell interactions between pathogen, host (Mourton et al. 1992, Ford and Alcox 1993), and gene function (Boulo et al. 1996).

Isolation of cells is the first and most important step for initiating primary cell culture and establishing a cell line. Viability, cell yield, and functional integrity of isolated cells need to be maximized during the process. Physical methods, chemical methods, or a combination of both have been used to isolate cells (Freshney 1994). Physical methods, including scraping, rubbing, and agitation (Momtazi and Herbert 1973), are used to break tissues into individual cells or to mince tissues into 1 to 2 mm^3 explants, from which individual cells can migrate. Chemical methods involve the addition of chelating agents, such as ethylenediaminetetraacetic acid (EDTA), to deplete divalent ions, which are

essential for adhesion between cells, and the addition of proteolytic enzymes, to digest extracellular matrices (Freshney 1994). Currently, tissue explant and enzyme dissociation are the two major methods used to isolate oyster cells for primary cell culture. Enzyme dissociation provides large amounts of different cell types immediately after dissociation, while tissue explants provide only a few cells after 7 to 14 days (d) of culture and yield only specific cell types that are able to migrate from the explant. For example, granulocytic hemocytes are the main cell type observed after 7 to 10 d using heart explants, while hemocytes, pigment cells, fibroblast-like cells and cardiomyocytes are the main cell types elicited from enzyme dissociation of heart cells (Wen et al. 1993, Le Deuff et al. 1994). The cell types derived from enzyme dissociation can enhance studies of the oyster immune system (Renault et al. 1995).

Enzymes, often used in dissociation of vertebrate tissues, have been used to dissociate oyster tissues. Pronase, trypsin, and collagenase were used to dissociate heart cells of the eastern oyster, and collagenase was more efficient than others in viable cells yields (Brewster and Nicholson 1979). Trypsin was effective in dissociating heart but not the mantle of eastern oyster (Hetrick et al. 1981). Collagenase and pronase were more efficient than trypsin for dissociating heart cells of the pacific oysters in terms of viable cells and cell attachment (Wen et al. 1993). Pronase was used to dissociate heart cells of pacific oysters and those cells possessed the ability to express a heat shock inducible transgene after dissociation (Boulo et al. 1996). Trypsin was used to dissociate heart cells of eastern oyster, pacific oyster and European flat oyster *Ostrea edulis*, for culture (Sami et al. 1991, Le Deuff et al. 1994, Renault et al. 1995).

The reports on the effectiveness of these enzymes varied, and no systematic comparison and statistical analyses were reported. The goal of this research was to systematically compare the efficiency of collagenase, pronase, and trypsin in dissociating atrium, ventricle, and mantle of the eastern oyster. The objectives were to: 1) dissociate atrium, ventricle, and mantle using collagenase, pronase, and trypsin and 2) compare total cell number, cell yield, viability, viable cell yield and the dehydrogenase activity of dissociated cells of each tissues.

Materials and Methods

Oyster Depuration and Tissue Decontamination

Eastern oysters, 9 to 10 cm in length, were obtained from reefs near Grand Isle, Louisiana. The shell surfaces were scrubbed vigorously with a 5% solution of Chlorox^{R} (5.25% sodium hypochlorite by weight), rinsed with fresh water, and maintained in a recirculating system with ultraviolet-treated artificial seawater (15‰ salinity, 25° C)(Fritz Super Salt, Friz Industries Inc. Dallas, Texas). Oysters were held without feeding one week prior to dissociation experiments to reduce microorganism loads (Hetrick et al. 1981).

Thirty oysters were opened. The oyster heart is located at the left side of the adductor muscle and is covered by a pericardial membrane (Appendix E). Thirty atria, 10 ventricles and 3 mantles were dissected, and washed three times with 50 ml of 0.45-μm membrane-filtered (Nalgene, Rochester, New York) artificial sea water (FASW). Each tissue type was transferred to 50-ml tubes containing 10 ml of decontamination solution (Appendix A), and the tubes were incubated 30 min at room temperature on a rocking platform (3 cycles/min) to reduce microorganism contamination (Hetrick and Stephens

1979, La Peyre et al. 1993). Decontaminated tissues were centrifuged at 200 X g for 3 min to remove FASW from the tissues. The SW was discarded and the tissues were weighed. The tissues were minced into 1 to 2 mm^3 pieces to facilitate cell dissociation.

Selection of Dissociation Enzymes and Solutions

The enzymes, collagenase (type XI, Sigma Chemical Corp, St. Louis, Missouri), trypsin (Sigma), and pronase (CalBiochem, La Jolla, California) were used because they contain other proteases or nonspecific enzymes that can facilitate digestion of cell matrices and cell junction proteins to release single cells. Collagenase type XI was used because it has higher collagenase and nonspecific neutral protease activity than other types. Saline solutions (saline I and saline II)(Appendix B) rather than culture medium were used as a dissociation solution to simplify conditions. Collagenase and pronase require divalent cations to function (Bond and Wart 1984, Narahashi et al. 1968), therefore saline I, which contains calcium and magnesium was used. Trypsin does not need divalent cations to function, so saline II was used. For facilitating cell dissociation, EDTA was added to saline II to deplete divalent cations, which are essential for cell-to-cell adhesion. The carbohydrates in saline II were used to prevent the re-aggregation of the dissociated cells and to enhance yields (Farris 1968).

Tissue Dissociation

Enzyme dissociation combined with physical agitation was chosen for the isolation of oyster cells, which are needed in large numbers for the establishment of cell lines and *in vitro* studies. Each type of minced tissue was equally divided into four 30-ml beakers, labeled A, B, C, and D. Saline I (9 ml) was added to beakers A, B, and C and saline II (9 ml) was added to beaker D. Thereafter, 1 ml of the following reagents was added for a

total volume of 10 ml in each beaker: saline I for beaker A (control), collagenase (750 U/ml, Sigma) for beaker B, pronase (450 U/ml, CalBiochem, La Jolla, California) for beaker C, and trypsin-EDTA (5000 U/ml trypsin, 0.2% EDTA w/v, Sigma) for beaker D. The beakers were placed on a stir plate (Model Nuova II, Thermolyne Corporation, Dubuque, Iowa) and octagonal stir bars (8 mm diameter x 12 mm long) were used to agitate the solution at 700 revolutions per minute (rpm) during dissociation. Tissues were dissociated for 60 min at 25° C, except for the trypsin-treated tissue, which was dissociated for 30 min. One ml of fetal bovine serum (FBS, Sigma) was added to beaker D to inactivate trypsin after dissociation. Any undissociated tissue was separated from dissociated cells in 10-ml conical tubes by centrifugation (Model TJ-6 centrifuge, Beckman, Palo Alto, California) at 20 x g for 3 min. The supernatants containing dissociated cells were transferred to 10-ml conical tubes and the undissociated tissue pellets were discarded. Dissociation enzymes were eliminated by centrifugation at 200 x g for 10 min, and the supernatants were discarded. These cell pellets were resuspended in saline II. Centrifugation and re-suspension of the pellets in saline II was repeated twice. Finally, the cell pellets were suspended in JL-ODRP-4 culture medium (Appendix C)(La Peyre et al. 1993) with 10% FBS for evaluation.

Evaluation Parameters

Total cell number, cell yield, cell viability, viable cell yield and dehydrogenase activity were evaluated. Total cell number was counted with a hemocytometer at 100 x magnification using phase-contrast microscopy (Optiphot-2, Nikon, Garden City, New York) and cell yield was calculated (total cell number divided by tissue weight). Cell viability was evaluated using a dye exclusion assay using trypan blue (Phillips 1973) and a

LIVE/DEAD® Assay Kit (Molecular Probes, Eugene, Oregon) that uses fluorescent dyes to indicate viability and cytotoxicity (referred to as fluorescent dye assay in this paper). Viable cell yield was calculated by multiplying cell viability and cell yield. The dehydrogenase activity was measured using a Cell Titer 96™ kit (Promega, Madison, Wisconsin) and the manufacturer's suggested protocol. This is a cell proliferation assay that is aqueous and non-radioactive.

Data Analysis

Tissue dissociation was repeated five times. Due to variability during each dissociation, data were analyzed using a randomized block design and one-factor analysis of variance (ANOVA). Duncan's multiple range test was performed when significant differences were found ($P < 0.05$).

Results

For atrial tissues, pronase treatment produced significantly higher ($P < 0.05$) total cell numbers ($13.8 \pm 7.1 \times 10^6$ cells, n = 10) and cell yields ($170.9 \pm 52.2 \times 10^7$ cell/g tissue) than did other treatments (Figure 2-1). No significant difference ($P > 0.05$) was found among tissues treated with collagenase, trypsin, and the control. Regardless of the method used, the pronase-dissociated cells had significantly higher viability (92 ± 4 % for the trypan blue assay, 89 ± 5% for fluorescent dyes assay) than did cells dissociated with collagenase, trypsin, or the control (Figure 2-2). Trypsin-dissociated cells had significantly higher viability ($P < 0.05$) than collagenase dissociated cells in a trypan blue dye assay (87 ± 3% vs. 85 ± 3%) and dehydrogenase activity assays ($44.0 \pm 11.35 \times 10^{-3}$ vs. $32.2 \pm 4.9 \times 10^3$). Pronase-treated tissues also had a significantly higher viable cell

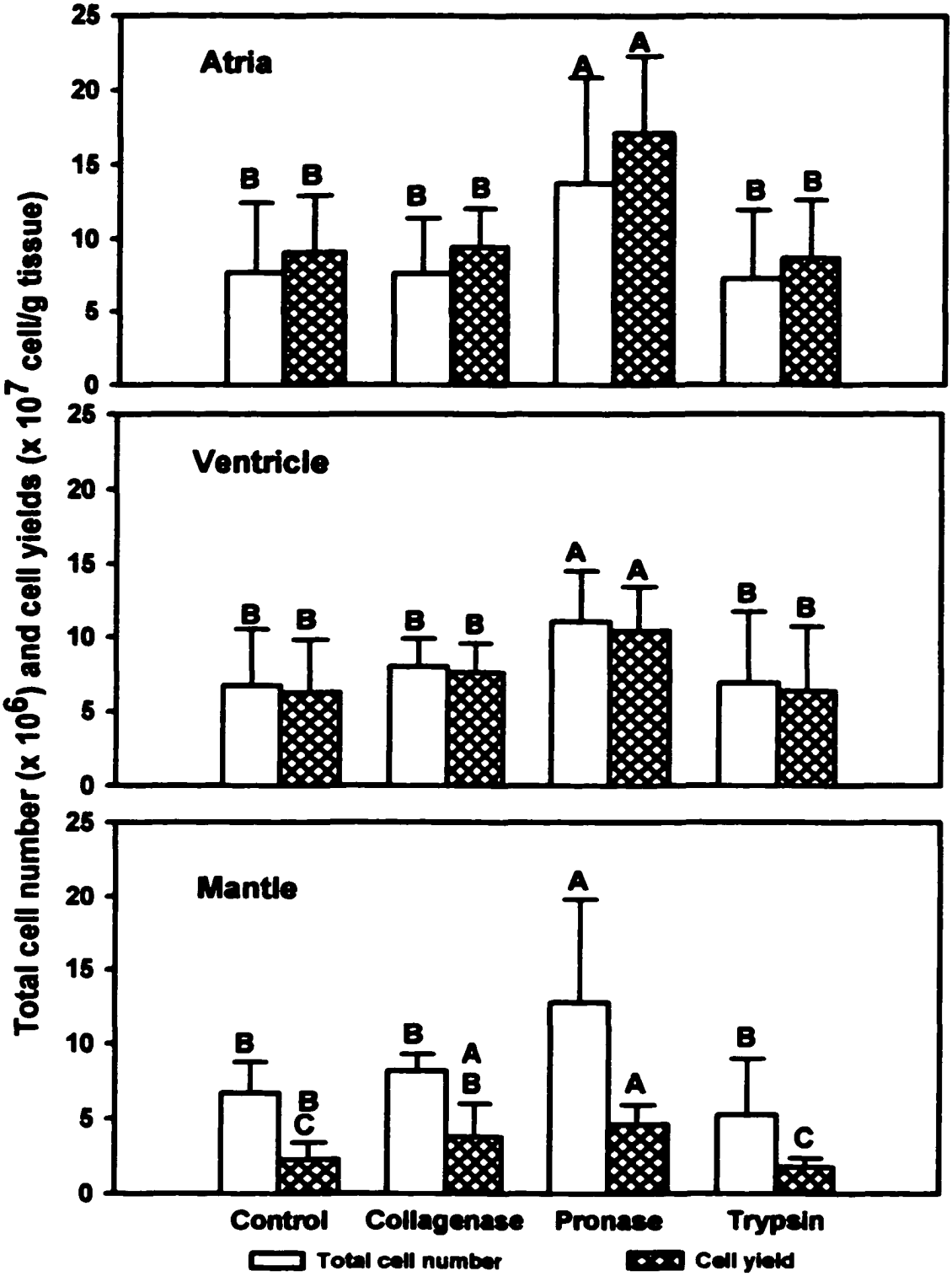

Figure 2-1. Total cell number and cell yield of enzyme-dissociated oyster tissues (n = 5 for each treatment). Controls were dissociated without enzyme. Means of each parameter sharing letters (A, B, and C) were not significantly different ($P > 0.05$).

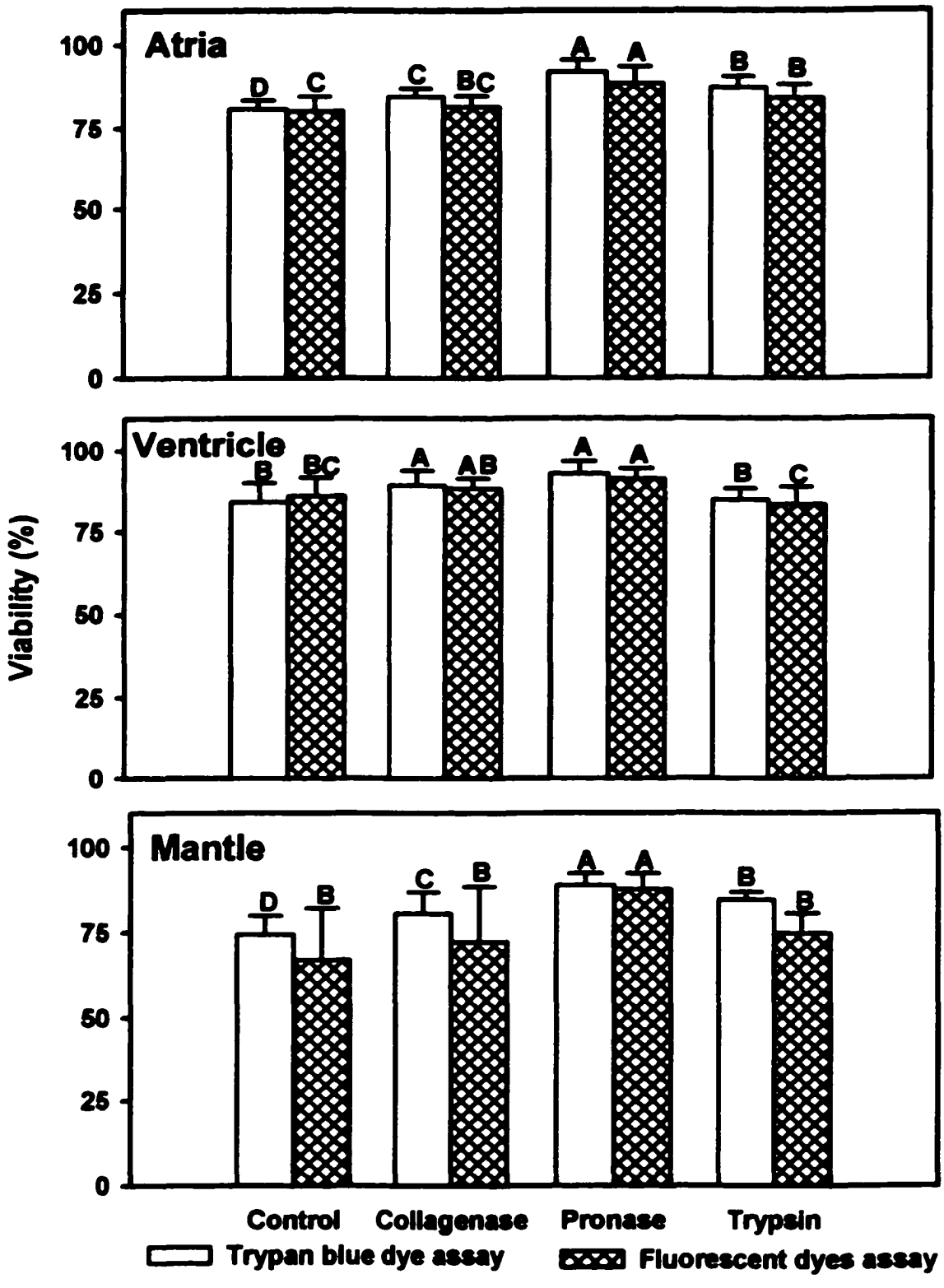

Figure 2-2. Viability of enzyme-dissociated oyster tissues (n = 5 for each treatment). Controls were dissociated without enzymes. Means of each assay sharing letters (A, B, C, and D) were not significantly different ($P > 0.05$).

yield ($156.7 \pm 45.6 \times 10^6$ cell/g tissue for the trypan blue assay, $149.8 \times 10^6 \pm 40.5$ cell/g tissue for the fluorescent dye assay (Figure 2-3), and dehydrogenase activity ($62.0 \times 10^{-3} \pm 7.9$ $A_{490\ nm}$) than did other treatments (Figure 2-4).

For ventricle tissues, pronase treatment produced a significantly higher ($P < 0.05$) total cell number ($11.0 \pm 3.5 \times 10^6$ cells) and cell yield ($103.9 \pm 30.7 \times 10^7$ cell/g tissue) than did other treatments (Figure 2-1). In viability assays, no significant difference ($P > 0.05$) was found between pronase-dissociated cells and collagenase-dissociated cells in a trypan blue dye assay (93% vs. 89%) and fluorescent dye assay (91% vs. 88%) (Figure 2-2) but in terms of viable cell yield, pronase-dissociated tissues generated a significantly higher yield ($96.4 \times 10^6 \pm 27.9$ cell/g tissue for trypan blue assay, $94.7 \pm 27.9 \times 10^6$ cell/g tissue for fluorescent dye assay) than did collagenase-dissociated tissues, trypsin-dissociated tissues and control tissues, regardless of the assay method used (Figure 2-3). In the dehydrogenase activity assay, pronase-dissociated cells showed a significantly higher dehydrogenase activity ($92.5 \pm 51.1 \times 10^{-3}$ $A_{490\ nm}$) than did other treatments (Figure 2-4).

For mantle tissues, pronase treatment generated significantly higher total cell numbers ($12.8 \pm 7.0 \times 10^6$ cells) than did other treatments, but in terms of cell yield, no significant difference was found between pronase-dissociated tissues and collagenase-dissociated tissues ($46.2 \pm 13.2 \times 10^7$ cell/g vs. $38.2 \times 10^7 \pm 21.8$ cell/g)(Figure 2-1). A significantly higher viability was found in pronase-dissociated cells (90% for trypan blue assay; 88% for fluorescent dye assay)(Figure 2-2). Meanwhile, a significantly higher viable cell yield was found in pronase-dissociated tissues ($41.2 \pm 12.4 \times 10^6$ cell/g for

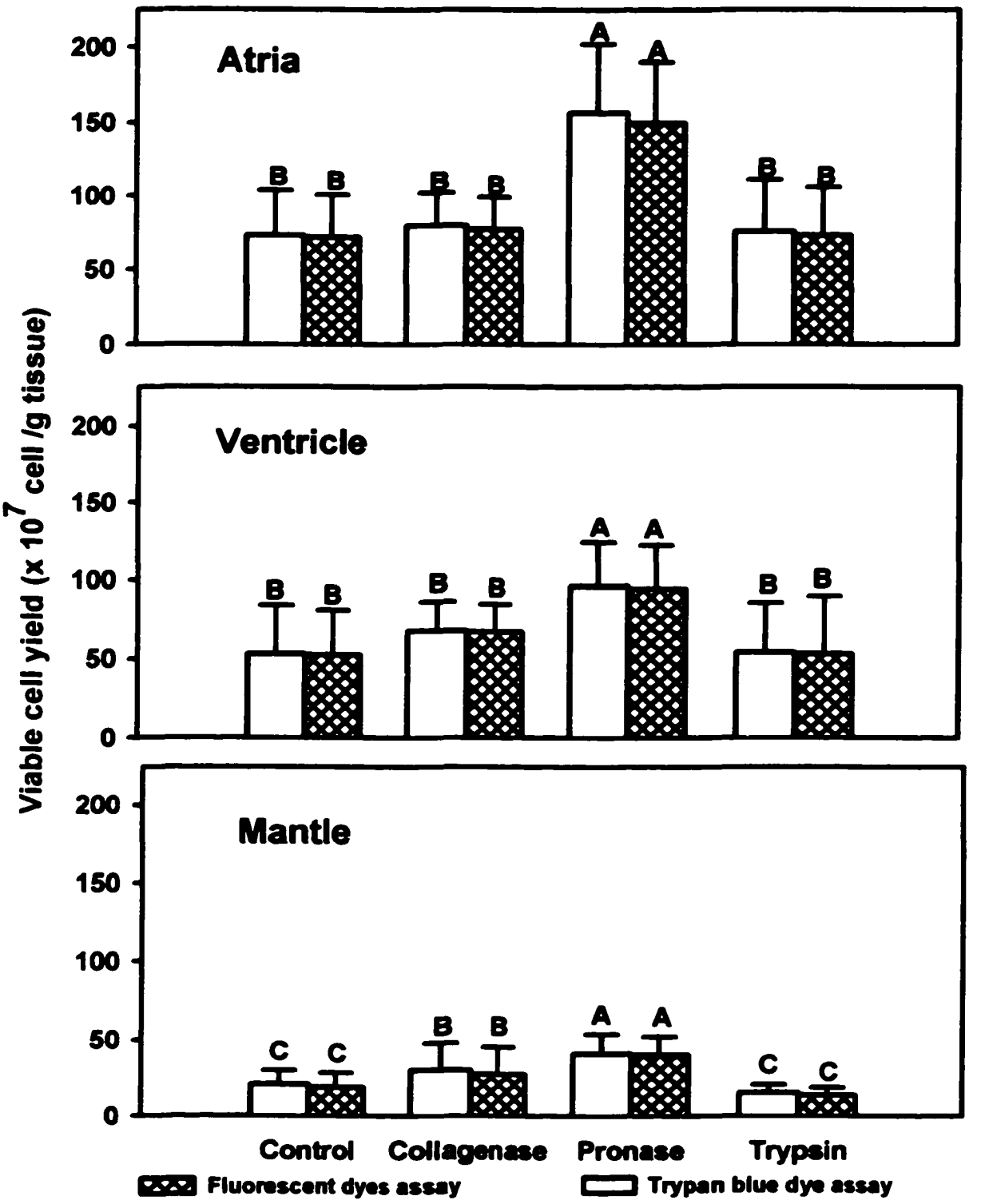

Figure 2-3. Viable cell yields of enzyme-dissociated oyster tissues(n = 5 for each treatment). Controls were dissociated without enzymes. Means of each assay sharing letters (A, B, and C) were not significantly different (P > 0.05).

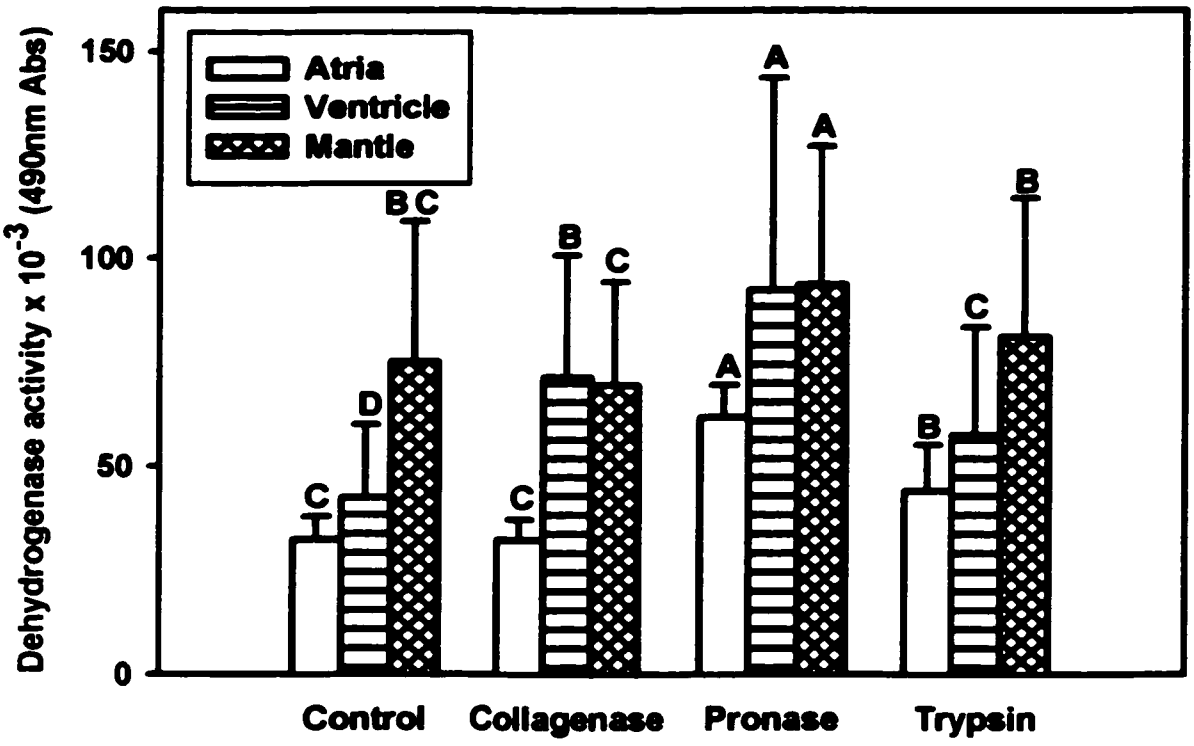

Figure 2-4. Dehydrogenase activity of enzyme-dissociated oyster tissues (n = 5 for each treatment). Controls were dissociated without enzyme. Means of each tissue sharing letters (A, B, C, and D) were not significantly different ($P > 0.05$).

trypan blue assay, 40.4 ± 11.62 x 10^6 cell/g for fluorescent dye assay)(Figure 2-3). A significantly higher dehydrogenase activity was found in pronase-dissociated cells (93.7± 33.10 x 10^{-3} $A_{490\ nm}$)(Figure 2-4). Although trypsin-dissociated cells showed significantly higher viability than collagenase-dissociated cells in the trypan blue assay (85 % vs. 81%) and dehydrogenase activity (81.5 ± 33.0 x 10^{-3} vs. 69.9 ± 24.6 x 10^{-3} $A_{490\ nm}$), collagenase-treated cells had a significantly higher viable cell yield than trypsin-treated cells.

Discussion

Although oyster tissues have been routinely dissociated with enzymes for *in vitro* studies and attempts have been made to establish cell lines, the most effective dissociation enzymes have not been previously identified due to a lack of systematic comparison and the evaluation of statistical data. This study represents the first systematic approach and statistical analysis of enzymatic-dissociated cells from atrium, ventricle, and mantle of the eastern oyster.

The dissociation efficiency of pronase has been suggested to be less than that of collagenase in dissociating heart cells of eastern oysters (Brewster and Nicholson 1979), but pronases can effectively liquefy mucins, which are the main component of mucus. Pronase has been used successfully to isolate feline goblet cells from mucus-rich trachea (Sherman et al. 1988) and rat gastric mucosal cells from the stomach (Kurokawa et al. 1975) while maintaining cell morphology, viability, and functional integrity of the mucous secretion. Therefore, pronase would be expected to be more efficient than collagenase or trypsin in dissociating mantle tissues, which are high in mucin content. Surprisingly, not only pronase-treated mantle, but also pronase-treated ventricle and atrium, generated

significantly higher viable cell yields and dehydrogenase activity. This suggests that oyster extracellular proteins and compounds are susceptible to pronase, and that other tissues may be dissociated effectively by pronase.

Because one of the major extracellular proteins in multicellular animals is collagen, collagenase is widely used to dissociate vertebrate tissues (Burgeson 1988). Collagenase has been suggested to be a more efficient enzyme than trypsin for dissociating heart cells of eastern oyster and Pacific oyster (Brewster and Nicholson 1979, Wen et al. 1993) but we found that collagenase is much less efficient than trypsin and pronase.

During dissociation, trypsin-treated atrial and ventricle tissues were aggregated with viscous materials. This may be due to the release of nucleic acids from lysed cells. Addition of deoxyribonuclease, or reducing the trypsin concentration, or incubation time may further improve the dissociation efficiency of trypsin. Regardless of the enzyme used, cell yields from mantle were lower than atrium, ventricle, and undissociated mantle tissues remained intact without viscous materials. This implied that mantle was not as susceptible as atrial and ventricle tissues to collagenase, pronase, and trypsin. Using a combination of pronase with other dissociation enzymes may increase the cell yield from mantle.

Unexpectedly, the control in atrium and ventricle dissociation showed no significant difference in total cell number, cell yield and viable cell yield when compared to samples treated with collagenase and trypsin. This indicates that if the dissociated cell yield and viability were not the main objective, oyster heart could be dissociated by mechanical agitation. These cells could then be used in studies without protease interference.

In the present study, trypan blue dye exclusion and fluorescent dye assays were used to evaluate cell viability. Dye exclusion using trypan blue tended to indicate higher viability but significant differences in values were not found between trypan blue dye and fluorescent dye assays. In the fluorescent dye assay, calcein was used to identify live cells and ethidium homodimer-1 (EthD-1) was used to identify dead cells. It appeared that EthD-1 could be used alone like trypan blue. The principle of EthD-1 entry into cells, is the same as that for trypan blue, but EthD-1 stains nucleic acids in the nucleus and with 40-fold increases in red fluorescence, which can be observed by fluorescent microscopy. The strong red fluorescence of EthD-1 stained cells can be distinguished more easily than the trypan blue-stained cells that may be obscured by stained extracellular proteins. Therefore, we concluded that viability evaluation using EthD-1 alone is easier, more accurate and more efficient than evaluation using trypan blue.

In conclusion, this is the first report of a quantitative comparison of enzymatic cell dissociation in oysters, and demonstration that pronase was the most efficient enzyme for dissociating atria, ventricle, and mantle tissues into single cells when measured by viable cell yield and dehydrogenase activity. Further optimization of dissociation efficiency may be achieved by using combinations of more than one enzyme. In our laboratory, pronase-isolated oyster cells are used for the development of a chemically defined medium, testing of lytic peptide toxicity, optimization of attachment factors, and transgene expression studies.

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CHAPTER 3 CRYOPRESERVATION OF HEART CELLS FROM THE EASTERN OYSTER

Introduction

Cryopreservation of cells is defined as maintenance at ultra-low temperatures to inhibit biological, chemical, and physiological processes of the cells (Karlsson and Toner 1996). Usually, cells are put into vials and stored in liquid nitrogen or above it, in the vapor phase. Major stresses such as the chemical and physical effects of ice crystals and osmotic shock can damage cells during freezing and thawing. However, these effects can be reduced by controlling the freezing rate, thawing rate, and by addition of chemicals such as dimethyl sulfoxide (DMSO)(Lovelock and Bishop 1959) and glycerol (Leueng 1991) as cryoprotectants to modify intracellular and intercellular environments. Cryopreservation has been used to store vertebrate cells to provide a constant source of cells for culture (Freshney 1994), and in addition, has the following advantages: 1) it is less expensive than maintaining live animals; 2) it minimizes the cost of cell culture maintenance; 3) it lowers the risk of viral, bacterial, and fungal contamination or accidental loss; 4) it reduces cell aging and genetic changes; and 5) it increases the ease of shipping to other laboratories. As a result, cryopreservation has become an indispensable tool for cell culture.

Cell culture is valuable for numerous *in vitro* studies. Cells can come from frozen cell lines (immortal lines) or freshly dissociated tissues (primary culture). Currently, an immortal oyster cell line is not available (McGladdery 1998). Primary cultures are the only cell source for *in vitro* studies. For instance, primary heart cell culture was used to study the function of the *Drososphila* heat shock gene promoter in oysters (Boulo et al. 1996) and hemocytes were used to study interactions with parasites (Ford et al. 1993,

Mourton et al. 1992). Unfortunately, with the exception of peripheral hemocytes, oyster primary cell cultures are obtained with a time-consuming (7 to 14 d) tissue explant culture method (Stephens and Hetrick 1979) or a labor-intensive enzyme digestion method (Renault et al. 1995). Moreover, oysters are filter feeders, accumulating large numbers of microorganisms, especially in the summer. Thus contamination is a major obstacle during cell isolation and culture. Although primary cultures of oyster heart cells and hemocytes (Wen et al. 1993, Stephens and Hetrick 1979) can be maintained for 1 to 6 months, the cost of culture maintenance is high, as is the risk of cell loss. Meanwhile, cell aging and genetic changes can interfere with research results. All of these problems make the establishment and use of primary cell cultures uncertain. Cryopreservation of cells can minimize these obstacles.

Cryopreservation conditions have been developed for bivalve germ line cells such as sperm and oocytes of the pacific oyster, *Crassostrea gigas*, (Yankson and Moyse 1991, Naidenko 1997). Cryopreservation conditions for embryos of, *C. gigas*, (Gwo 1995), hard clam, *Meretrix lusoria*, (Chao et al. 1997) and eastern oyster, *C. virginica*, (Paniagua et al. 1998) were established. For dissociated cells such as the mantle and the gill of mussel, *Mytilus trossulus*, digestive gland cells of the scallop, *Mizuchopecten yessoensis*, (Odintsova and Tsal 1995) and heart of the scallop, *Pecten maximus*, (Le Marrec-Croq et al. 1998), cryopreservation conditions were also reported. However, effective cryopreservation conditions vary with each species, cell type, and developmental stage. Cryopreservation conditions for dissociated oyster cells have not been reported. The goal of this work was to develop cryopreservation conditions for oyster heart cells, which are considered to be the best tissue for *in vitro* studies (Chen and

Wen 1999). The objectives were to compare the effects of: 1) cryoprotectant type and concentration; 2) freezing rate; 3) thawing rate; and 4) each cryoprotectant on cryopreservation of pronase-dissociated atrial and ventricle cells of the eastern oyster.

Materials and Methods

Oyster Depuration

Oysters were collected at Grand Isle, Louisiana and maintained at the Department of Veterinary Science, Louisiana State University Agricultural Center, in a recirculating system containing ultraviolet-light treated 20 ‰, 25° C artificial seawater (ASW) that was prepared using Instant Ocean® salts (Aquarium Systems, Mentor, Ohio). Oysters were kept in the tank at for at least 1 week without being fed, to reduce the bacterial load.

Tissue Dissection and Decontamination

The oyster heart is located at the left side of the adductor muscle and is covered by a pericardial membrane (Appendix E). Ventricles and atria of 30 oysters were dissected and washed three times with 30 ml of ASW, which was filtered (FASW) with a 0.22-μm pore size membrane (Nalgene International, Rochester, New York). All chemicals were reagent grade and were purchased from Sigma Chemical Company (St. Louis, Missouri) unless otherwise indicated. Tissues were decontaminated as described in Chapter 2)

Tissue Dissociation

Ventricles and atria were dissociated separately in 50 ml beakers containing 30 ml of saline I (Appendix B) containing 45 U pronase/ml (Calbiochem, La Jolla, California) for 60 min. The beakers were placed on a stir plate (Model Nuova II, Thermolyne Corporation, Dubuque, Iowa) and octagonal stir bars (8 mm diameter x 12 mm long)

were used to agitate the solution at 700 revolutions per minute during dissociation. Any undissociated tissue was separated from cells in 50-ml conical tubes by centrifugation (Model TJ-6 centrifuge, Beckman, Palo Alto, California) at 20 x g for 3 min. The supernatants containing dissociated cells were transferred to 50-ml conical tubes and the undissociated tissue pellets were discarded. To remove pronase, the cells were pelleted by centrifugation at 200 x g for 10 min. The supernatants were discarded and cells were resuspended in 30 ml of saline II (Appendix B); this was repeated twice. After the last centrifugation, the cell pellets were resuspended in 10 ml of freezing medium, which contained JL-OPRD-4 medium (La Peyre et al. 1993) without antibiotics and with 20% (v/v) fetal bovine serum (FBS) and a cryoprotectant additive (refer to Study 1 below). Cell numbers were counted with a hemocytometer (Freshney 1994) and the cell density was adjusted to 5 x 10^6 cell/ml in the same freezing medium used for suspension and counting.

Study 1: Comparison of Cryoprotectants and Concentrations

The effects of dimethyl sulfoxide (DMSO), glycerol, and propylene glycol (PG) on dehydrogenase activity and cell spreading of atrial and ventricle cells were evaluated at 5%, 10% and 15% final concentrations. All procedures before freezing at –80° C were performed at 25° C unless otherwise indicated. Each treatment had 3 replicates and medium containing no cryoprotectant was used as a control. Based on preliminary studies of determining the initial freezing rate, the cryopreservation conditions were defined in three comprehensive steps:

1) Addition of cryoprotectants and cell freezing: An equal volume of atrial or ventricle cells at 5 x 10^6 cell/ml and freezing medium containing a 2 X final

concentration of cryoprotectant were mixed. The mixture was distributed into 2-ml cryovials (Sarstedt, Leicester, United Kingdoms) in 700 µl aliquots for freezing. Cryovials containing atrial cells were packed into a 21 x 19 x 7 cm and 0.7 cm thick polystyrene foam box and wrapped with 2 layers of cotton (80 g/layer). Cryovials containing ventricle cells were packed into a polystyrene foam box without additional wrapping. Atrial and ventricle cells were equilibrated at 25° C for 20 min, at -80° C for 16 h, and plunged into liquid nitrogen (-196° C) for storage.

2) Cell thawing and cryoprotectant removal: After 3 d of storage, cells in individual cryovials were thawed in a 45° C water bath with shaking until all ice crystals had melted (~ 1 min). The thawed cell suspension was gently transferred to a 15-ml conical tube. Culture medium was added drop-by-drop, over 20 min until the freezing medium was diluted 10 folds, and the cells were pelleted by centrifugation at 4° C at 200 x g for 10 min. The supernatants were discarded and the cells resuspended in 700 µl of culture medium and seeded for culture at a volume of 100 µl/well in a 96-well plate (Falcon Plastics, Becton Dickinson Inc., Franklin Lakes, New Jersey).

3) Evaluation of dehydrogenase activity and extent of cell spreading: Cells were cultured for 3 d at 25° C. Dehydrogenase activity of mitochondria was used as an index of cell viability (Mosmann 1983). The dehydrogenase activity (absorbance at 490 nm, A_{490}) was assessed using a Cell Titer 96™ aqueous non-radioactive cell proliferation assay kit (Promega, Madison, Wisconsin) following the manufacturer's protocol.

With a phase-contrast inverted microscope (Diaphot-TMD, Nikon, Garden City, New York) at 400X magnification, the extent of cell spreading was ranked. Cells that were flattened and extended were counted as "spread." The ratio of the number of spread

cells to total cell numbers from at least 300 cells was calculated as the percentage of cell spreading. The control that received no cryoprotectant and no freezing or thawing was ranked as the highest (100%) extent of cell spreading with "++++." By comparing with the control, the extent of cell spreading of other treatments were ranked as "+" (≤ 25%), "++" (> 25% but < 50%), "+++" (≥ 50% but < 75%), or "++++" (≥ 75%). The highest cell dehydrogenase activity and level of cell spreading for each treatment in the same cryoprotectant were used as criteria for selection of conditions for subsequent studies.

Study 2: Comparison of Thawing Temperature

The most effective concentration of each cryoprotectant in Study 1 was adopted for comparison of thawing temperatures for atrial and ventricle cells. Cryopreservation conditions and evaluation criteria were the same as in Study 1 except that thawing temperatures of 25° C, 45° C, and 70° C, were tested.

Study 3: Comparison of Freezing Rates

For further optimization of cryopreservation conditions, freezing rates were compared. Using data from Study 2, the most effective thawing temperature of each cryoprotectant was adopted for comparison of the freezing rate effect on atrial and ventricle cells. Cryopreservation conditions and evaluation criteria were the same as in Study 2 except for the freezing rates. Atrial cells were packed into a polystyrene foam box with four layers of cotton (slow freezing rate), two layers of cotton (medium freezing rate), or no cotton (fast freezing rate). Ventricle cells were packed into a polystyrene foam box (slow freezing rate), freezer paper box (medium freezing rate, 13 x 13 x 4.5 cm, Sarstedt), or without packing (fast freezing rate).

Study 4: Comparison of Optimal Conditions of Each Cryoprotectant

Based on Study 3, the optimal conditions for each cryoprotectant were determined for cryopreservation of atrial and ventricle cells. In addition to dehydrogenase activity and extent of cell spreading, cell viability was evaluated using a LIVE/DEAD® viability/cytotoxicity kit (Molecular Probes, Inc. Eugene, Oregon) according to the manufacturer's protocol. The number of cells recovered was counted with a hemocytometer after detachment from the wells with trypsin (Freshney 1994). All data were compared with data obtained from cells receiving no cryoprotectant or freeze-thawing steps. The percentages of dehydrogenase activity, cell viability, and cell recovery were calculated.

Statistical Analysis

The data for dehydrogenase activity were analyzed by two-factor analysis of variance in Study 1 (cryoprotectants and concentrations), Study 2 (cryoprotectants and thawing temperatures), and Study 3 (cryoprotectants and freezing rates) and the data for dehydrogenase activity, cell viability, and total cell number were analyzed by one-factor ANOVA in Study 4 (cryoprotectants). Whenever a significant difference was found ($P < 0.05$), the Tukey multiple means comparison was performed.

Results

Study 1: Comparison of Cryoprotectants and Concentrations

Cryoprotectant type and concentration significantly affected dehydrogenase activity of atrial and ventricle cells. Significant interaction was observed between cryoprotectant type and cryoprotectant concentration for cryopreserved atria ($P = 0.0001$)

and ventricle cells ($P = 0.0001$)(Figure 3-1). Compared with non-cryopreserved atrial cells (267 ± 20, A_{490}), a 74% reduction of dehydrogenase activity was found for atrial cells which were cryopreserved with 10% DMSO (96 ± 6 A_{490}). A 53% reduction of dehydrogenase activity compared to non-frozen ventricle cells (196 ± 5 A_{490}) was found for ventricle cells, which were cryopreserved with 10% glycerol (92 ± 8 A_{490}).

In the evaluation of cell spreading for each cryoprotectant treatment, atrial cells tended to form clumps in all cryoprotectant treatments. The ranked order of cell spreading for each cryoprotectant treatment was 10% glycerol (++++), 10% DMSO (+++) and 10% (++) PG. Cells that received no cryoprotectant showed the least cell spreading (+). For ventricle cells, less clumping was found in all cryoprotectant treatments. The ranked order of cell spreading for each cryoprotectant treatment was 10% glycerol (+++), 10% DMSO (++) and 10% PG (++). Cells receiving no cryoprotectant showed the least cell spreading (+). Based on the dehydrogenase activity and extent of cell spreading, 10% of each cryoprotectant was selected for Study 2.

Study 2: Comparison of Thawing Temperature

The thawing temperature did not significantly affect dehydrogenase activity of atrial cells that were treated with DMSO, glycerol or PG (Figure 3-2). No interaction was found between cryoprotectants and thawing temperatures ($P = 0.3159$). Regardless of the cryoprotectant, dehydrogenase activity of atrial cells thawed at 45° C (73 ± 25 A_{490}) was significantly higher than that of cells thawed at 25° C (67 ± 27 A_{490}) or at 70° C (65 ± 27 A_{490}) and no significant difference was found for cells thawed at 25° C and 70° C. Regardless of thawing temperature, significantly different ($P < 0.05$) dehydrogenase activities were found among atrial cells treated with DMSO (67 ± 9 A_{490} nm), glycerol

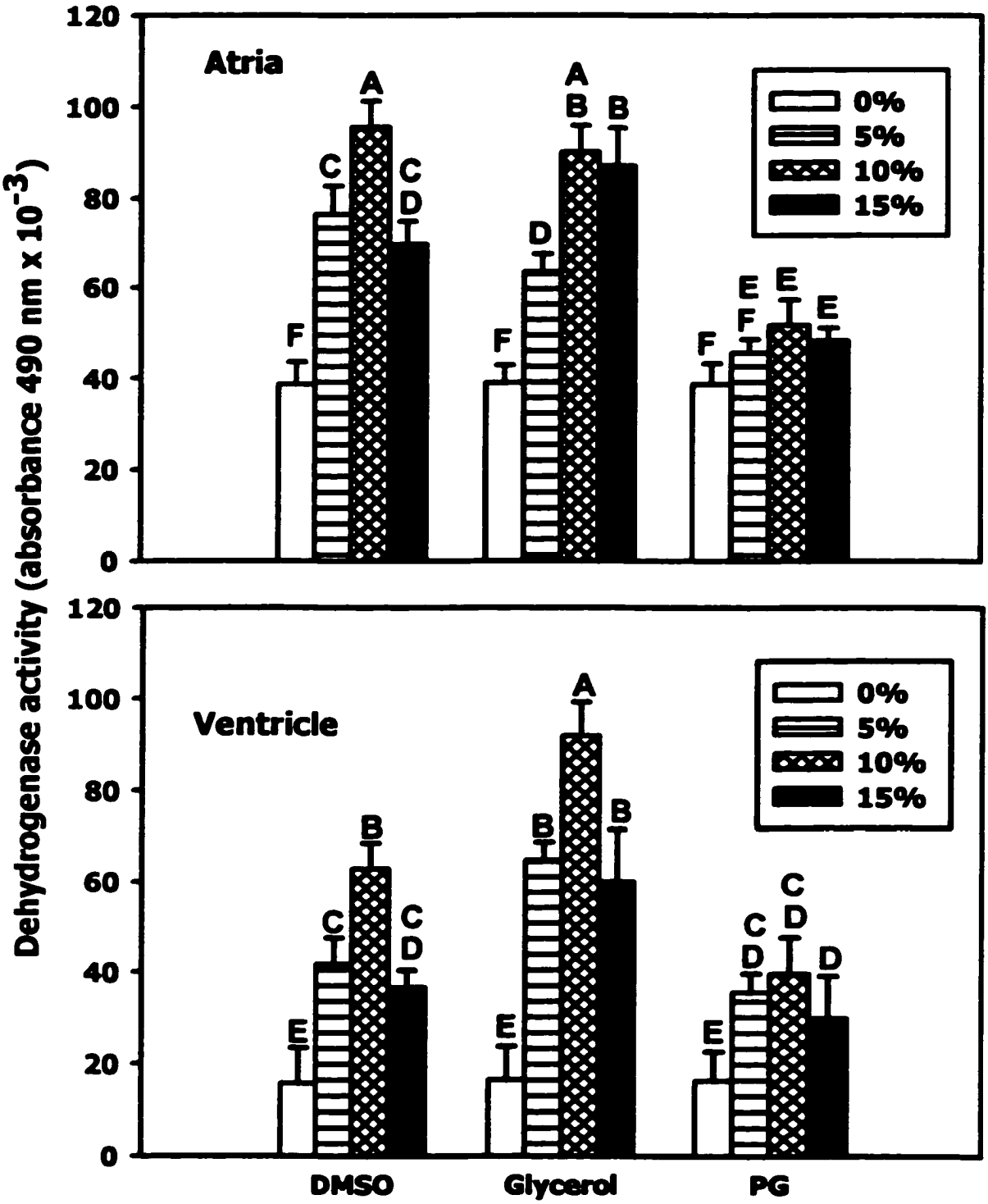

Figure 3-1. The effect of cryoprotectant and concentration on dehydrogenase activity of atrial and ventricle cells. Cells were frozen with dimethyl sulfoxide (DMSO), glycerol, and propylene glycol (PG) at three concentrations and thawed at 45° C. Dehydrogenase activities of thawed cells were evaluated after 3 d of culture. Bars sharing letters (A, B, C, D, E, and F) were not significantly different ($P > 0.05$).

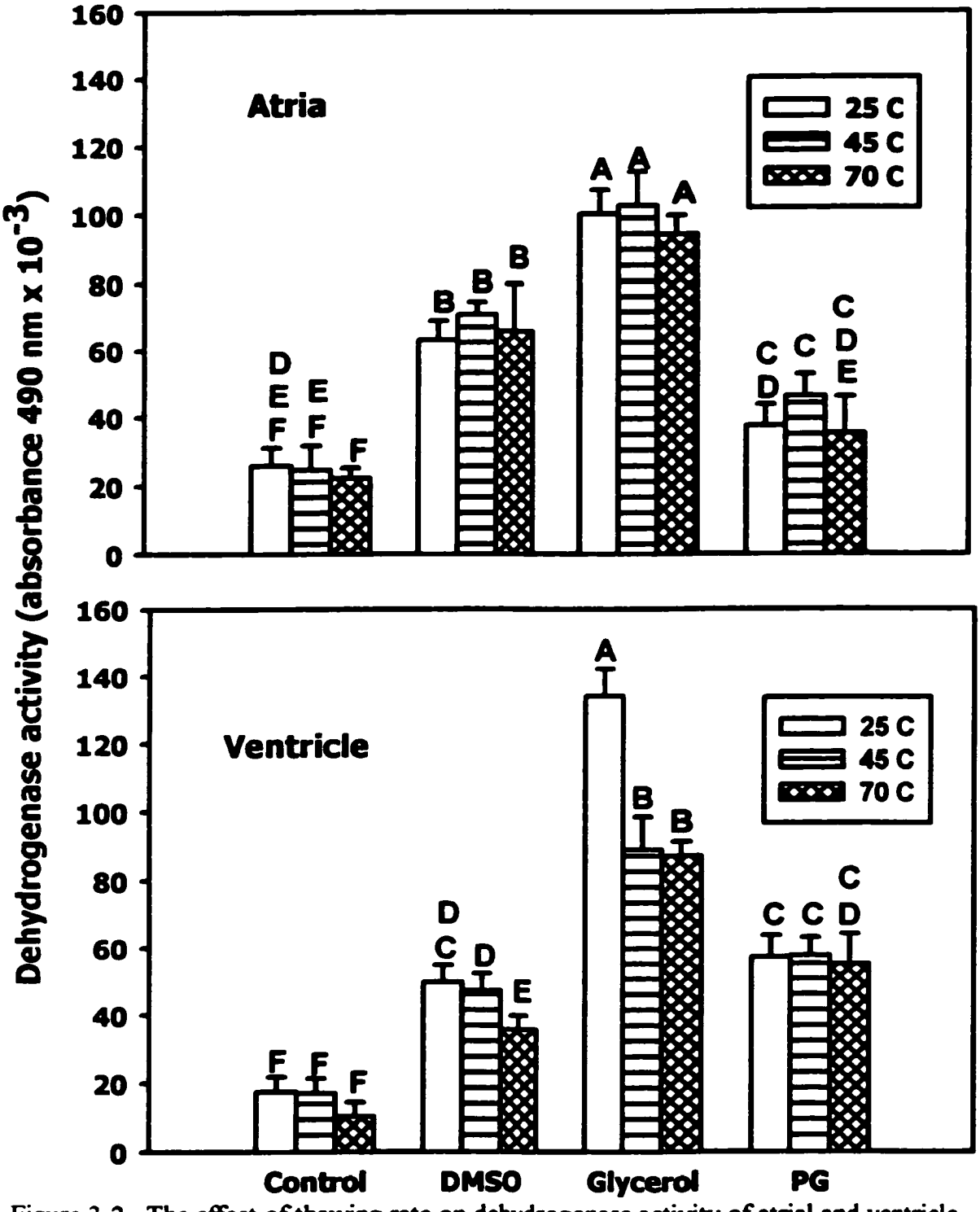

Figure 3-2. The effect of thawing rate on dehydrogenase activity of atrial and ventricle cells. Cells were frozen with a 10% final concentration of glycerol, dimethyl sulfoxide (DMSO), and propylene glycol (PG), and were thawed at three temperatures. The control indicates that cells were frozen without cryoprotectant. Dehydrogenase activities of cells were evaluated after 3 d of culture. Bars sharing letters (A, B, C, D, E, and F) were not significantly different ($P > 0.05$).

(99 ± 8 A_{490}), and PG (40 ± 9 A_{490}). A 69% decrease, compared with non-frozen atrial cells (336 ± 3 A_{490}), was found for atrial cells cryopreserved with 10% glycerol and thawed at 45° C (103 ± 10 A_{490}).

For ventricle cells, an interaction was found between thawing temperature and cryoprotectant ($P = 0.0001$). The highest dehydrogenase activity (134 ± 8 A_{490}), which was 40% of the dehydrogenase activity found in non-cryopreserved ventricle cells (333 ± 4 A_{490}), was observed in ventricle cells cryopreserved with 10% glycerol and thawed at 25° C (Figure 3-2).

Thawing temperatures did not affect atrial cell spreading in each cryoprotectant treatment but did affect ventricle cell spreading. The best ventricle cell spreading (++++) was found in cells frozen with 10% glycerol and thawed at 25° C. Based on these results, thawing temperatures of 45° C for atrial cells and 25° C for ventricle cells were selected for Study 3.

Study 3: Comparison of Freezing Rates

There were significant interactions between cryoprotectant and freezing rate (for atrial cells, $P = 0.0001$; for ventricle cells, $P = 0.0001$). The highest dehydrogenase activity (92 ± 7 A_{490}), which was 63% of that of the non-cryopreserved atrial cells (146 ± 10 A_{490}), was found in cells treated with 10% glycerol and frozen at the medium rate (Figure 3-3). The same trend was seen in ventricle cells for which dehydrogenase activity (78 ± 18 A_{490}) was 44% of that of the non-cryopreserved cells (179 ± 15 A_{490}).

The best atrial cell spreading was found at the fast freezing rate for the DMSO treatment (+++), and the medium freezing rate for the glycerol treatment (++++) and the PG treatment (++). For ventricle cells, the best cell spreading was found with the

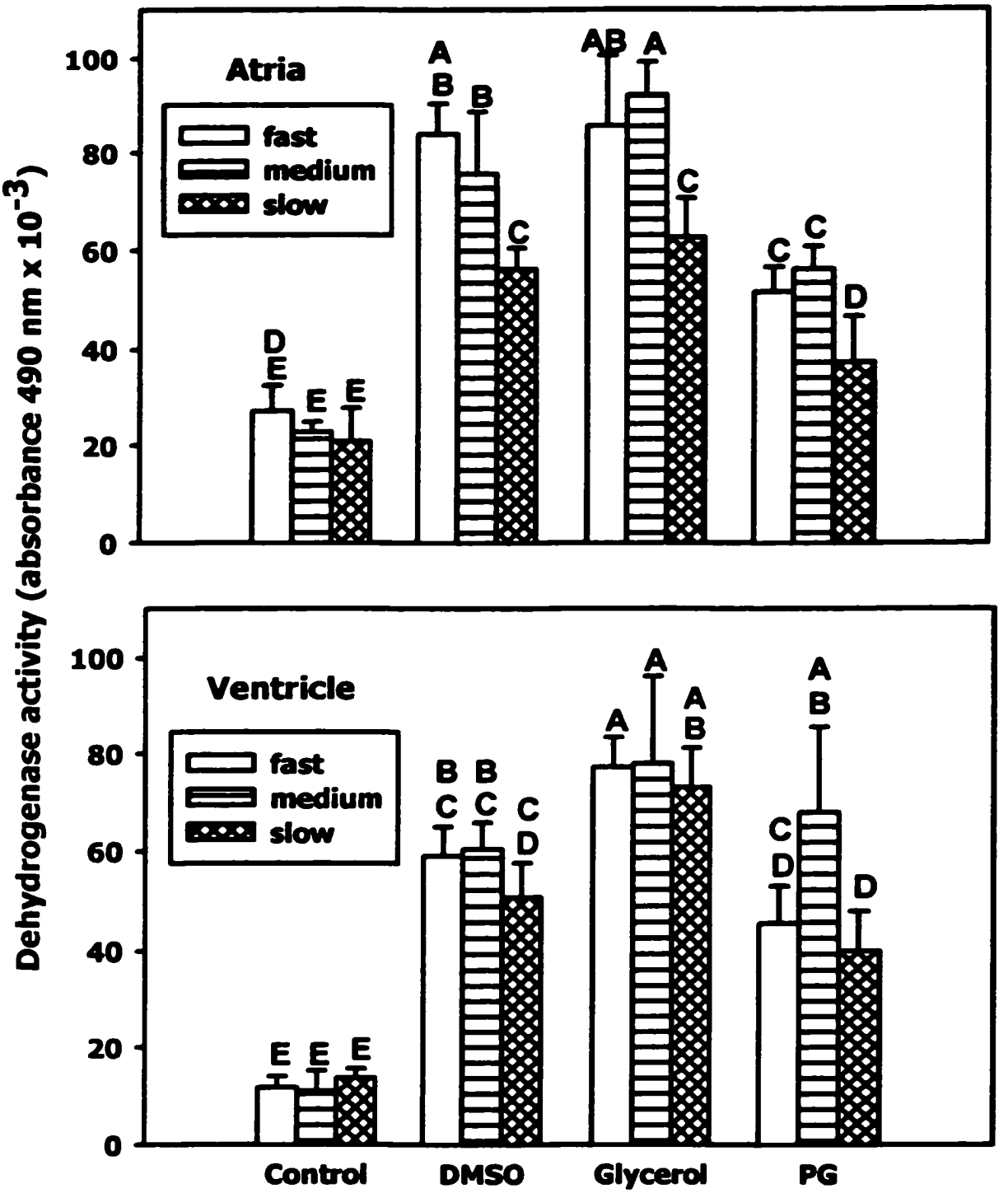

Figure 3-3. The effect of freezing rate on dehydrogenase activity of atrial and ventricle cells. Cells were frozen with 10% of glycerol, dimethyl sulfoxide (DMSO) and propylene glycol (PG) at three freezing rates. The control indicates that cells were frozen without cryoprotectant. Atrial cells were thawed at 45° C and ventricle cells were thawed at 25° C. Dehydrogenase activity of cells was evaluated after 3 d of culture. Bars sharing letters (A, B, C, D, and E) were not significantly different ($P > 0.05$).

medium freezing rate for the DMSO treatment (++), the glycerol treatment (++++) and the PG treatment (++). Based on the highest dehydrogenase activity and cell spreading, the following conditions were chosen for Study 4: for atrial cells, a fast freezing rate using 10% DMSO and a medium freezing rate using 10% glycerol and 10% PG; for ventricle cells, a medium freezing rate was selected.

Study 4: Comparison of Optimal Conditions of Each Cryoprotectant

The highest percentages of cell dehydrogenase activity, recovery, and viability were found in glycerol-treated atrial and ventricle cells (Figure 3-4). Regardless of the cryoprotectant, atrial cells tended to clump (Figure 3-5b, c, and d). This phenomenon was also found in control cells that received no cryoprotectant or cryopreservation (Figure 3-5a). Fragments of lysed cells were found in PG-treated cells (Figure 3-5d). The best spreading atrial cells were found with glycerol (++++) followed by DMSO (+++) and PG (++). Clumps were also formed in ventricle cells and control cells that received no cryoprotectant or cryopreservation (Figure 3-6). Fragments of lysed cells were found in DMSO-treated and PEG-treated ventricle cells (Figure 3-6b and 3-6d). The best cell spreading of ventricle cells was found in glycerol-treated cells (++++)(Figure 3-6c).

Discussion

In vitro studies of vertebrates have greatly enhanced the availability of cryopreserved cells. For oysters, *in vitro* studies are hampered due to the lack of cryopreservation protocols for cells and cell lines. In the present study, the first effective conditions for cryopreservation of atrial and ventricle cells of oysters were developed.

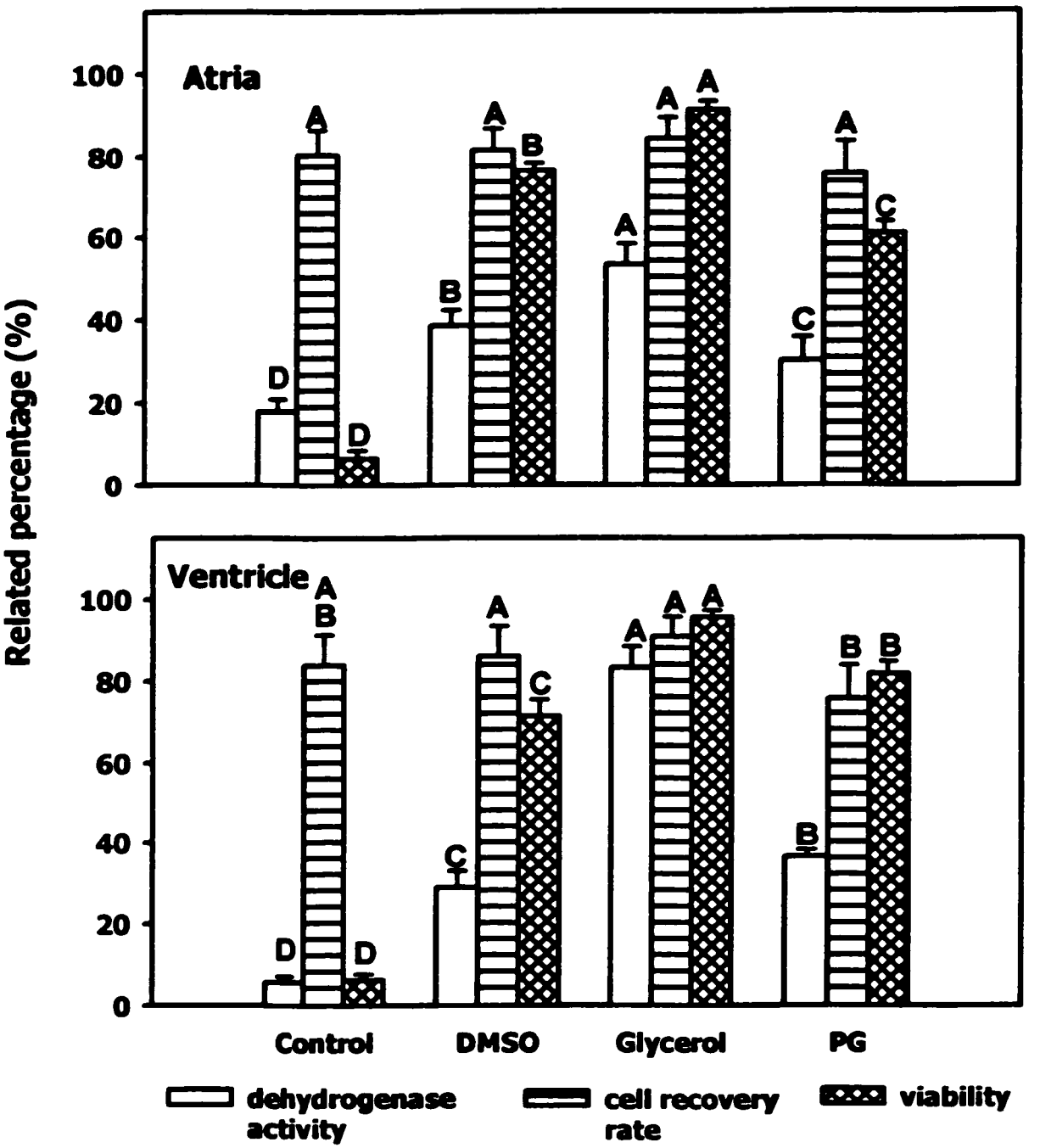

Figure 3-4. Comparison of the percentages of cell dehydrogenase activity, cell recovery rate and viability of atria and ventricle cells after thawing. The control indicates that cells were frozen without cryoprotectant (glycerol, dimethyl sulfoxide, DMSO, and propylene glycol, PG). These percentages were compared to those of untreated cells that received no cryoprotectant and cryopreservation. Cells were thawed at the optimal condition as follows: for atrial cells with 10% cryoprotectant, freezing at a medium rate and thawing at 45° C; for ventricle cells with 10% cryoprotectant, freezing at a medium rate and thawing at 25° C. Bars sharing letters (A, B, C, and D) were not significantly different ($P > 0.05$).

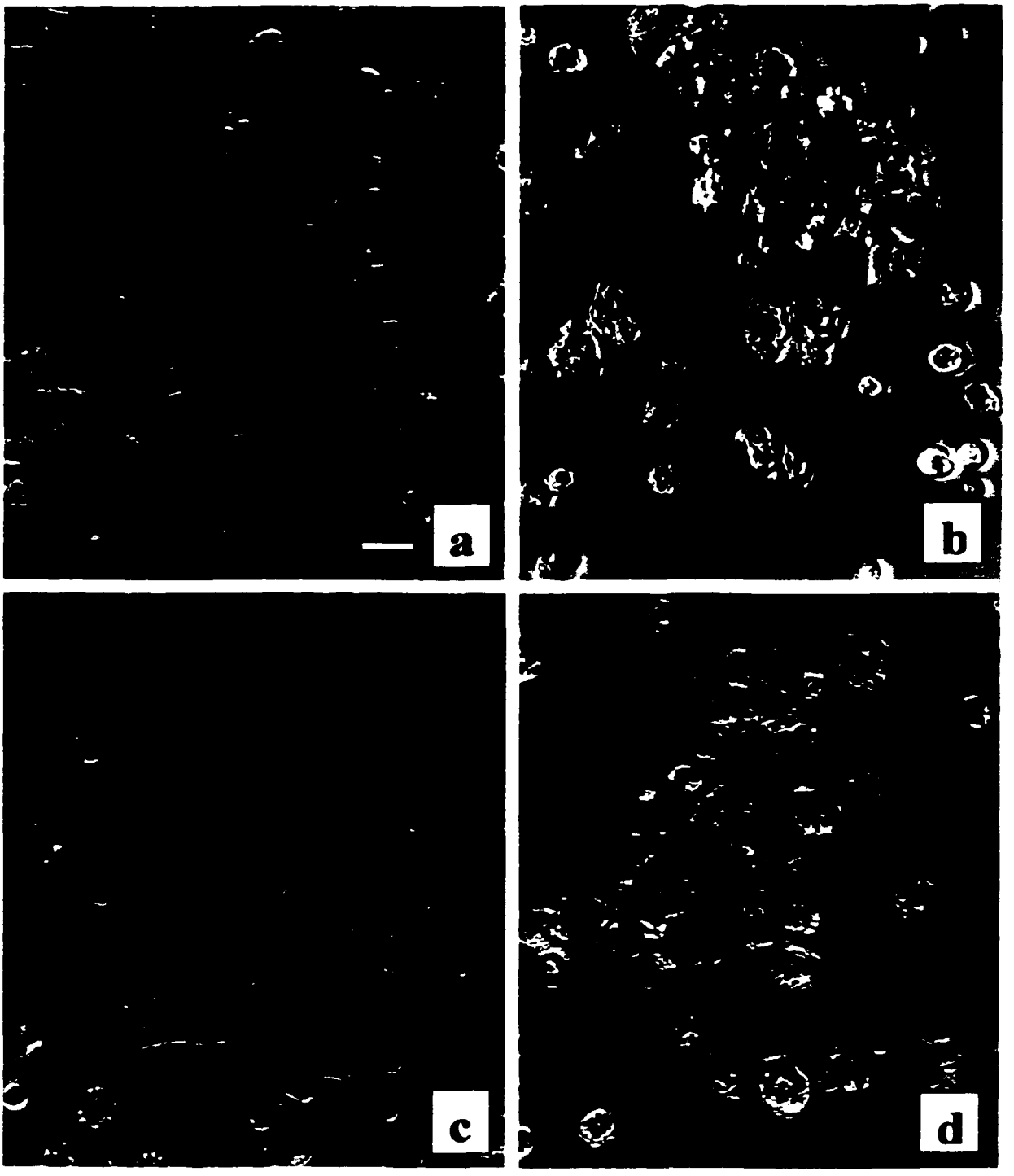

Figure 3-5. The spreading of thawed atrial cells after 3 d of culture. Cells were thawed at optimal conditions (10% cryoprotectant, freezing at a medium rate and thawing at 45° C). (a) Untreated cells which received no cryoprotectant and no cryopreservation; (b) cells treated with 10% dimethyl sulfoxide; (c) cells treated with 10% glycerol, and (d) cells treated with 10% propylene glycol. Bar = 20 μm.

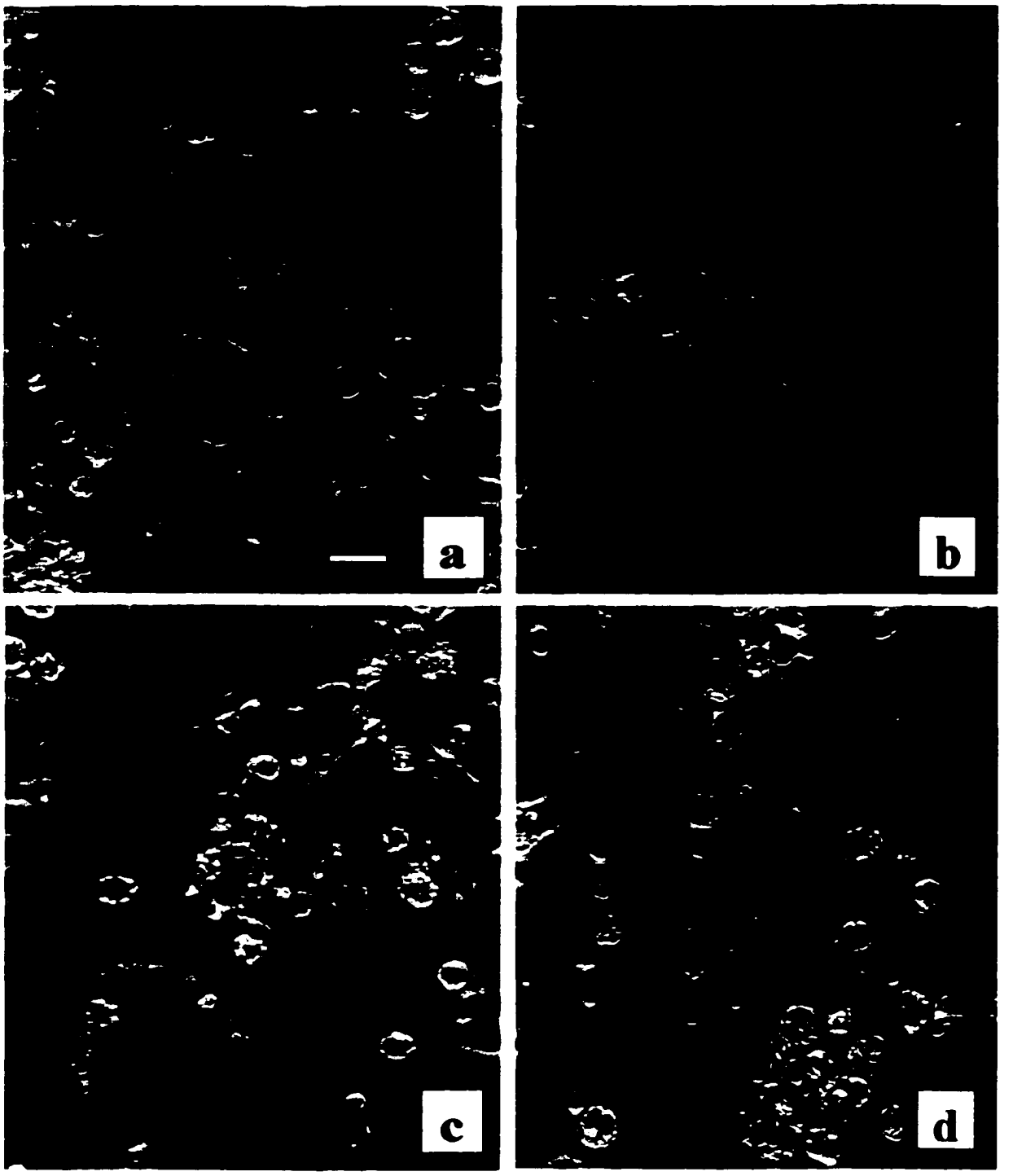

Figure 3-6. The spreading of thawed ventricle cells after 3 d of culture. Cells were thawed at optimal conditions (10% cryoprotectant, freezing at a medium rate and thawing at 25° C). (a) Untreated cells which received no cryoprotectant and no cryopreservation; (b) cells treated with 10% dimethyl sulfoxide; (c) cells treated with 10% glycerol, and (d) cells treated with 10% propylene glycol. Bar = 20 μm.

Osmotic shock and physical injury from ice crystals are two major factors causing cell death during freezing and thawing (Leung 1991). Addition of cryoprotectants, and optimization of freezing and thawing rates can minimize the damage to cells. Based on this study, cryopreservation of a cell line will be possible once it is established. Meanwhile, a frozen source of primary oyster cells will help to avoid problems with seasonal contamination.

Dimethyl sulfoxide is more toxic to animal cells than is glycerol but it can penetrate cell membranes faster due to its smaller molecular weight (Leung 1991). At a specific concentration, decreasing the exposure time or increasing the freezing rate can minimize the toxicity of DMSO to cells. In this study, atrial cells treated with 10% DMSO and frozen at a fast rate showed a higher dehydrogenase activity than that of cells frozen at medium and slow rates. In cryopreserving animal cells, glycerol has not been used as often as DMSO (Freshney 1994) because glycerol is viscous and difficult to remove after thawing. Glycerol-treated oyster cells consistently showed better results in all preliminary studies (data not shown) as well as in the four studies described here in terms of dehydrogenase activity, cell spreading, cell viability and number of cells recovered. The results demonstrated that glycerol-treated oyster heart cells tolerated a wider range of freezing and thawing rates than did DMSO-treated and PG-treated cells. These results suggest that glycerol is a useful cryoprotectant for oyster heart cells.

Although the PG was found to be an effective cryoprotectant for embryos of *C. gigas* (Gwo 1995) and trochophore larvae of *C. virginica* (Paniagua-Chavez et al. 1998), neither PG-treated atrial nor ventricle cells showed better dehydrogenase activity and cell spreading than did DMSO-treated or glycerol-treated cells. These results suggest that the

effective cryoprotectant for sperm, embryos and larvae may not be the same as for dissociated somatic cells of embryos.

During thawing, small ice crystals, which form during freezing, can grow and cause physical injury to cells (Forsyth and MacFarlane 1986). Control of thawing temperature can reduce damage due to ice crystals and increase cell viability. For ventricle cells, a 25° C thawing temperature enhanced dehydrogenase activity in glycerol-treated cells. However, thawing temperature was not critical for atrial cells regardless of cryoprotectant. This implies that a 37° C thawing temperature, used for mammalian cells, could be used for thawing atrial cells.

Further improvement of cryopreservation conditions may be achieved by inducing endogenous stress proteins such as heat shock proteins. Most heat shock proteins function as chaperones, which assist protein folding and conformation stability during stress (Morimoto 1993). For example, the viability of human fibroblasts was increased from 4% to 43% by heat shock treatment before hypothermic storage (Russotti et al. 1996). Heat shock proteins have been identified in *C. gigas* (Clegg et al. 1998). Inducing these endogenous stress proteins before freezing may enhance the tolerance of oyster cells, embryos and larvae.

In conclusion, the first effective cryopreservation conditions for oyster heart cells were developed as follows: for atrial cells, use of 10% glycerol, freezing at a medium rate, and thawing at 45° C; for ventricle cells, use of 10% glycerol, freezing at a medium rate, and thawing at 25° C. These conditions will improve oyster *in vitro* studies due to the availability and uniformity of cryopreserved oyster cells. Meanwhile they provide

basic information for further development of cryopreservation conditions for oyster cell lines when they are established.

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CHAPTER 4 *IN VITRO* EVALUATION OF INDUCIBLE HETEROLOGOUS PROMOTER FUNCTION IN THE EASTERN OYSTER

Introduction

Oyster culture has been plagued by diseases worldwide (Di Salvo et al. 1978, Hine et al. 1992, and Doonan et al. 1994). *Haplosporidium nelsoni*, the causative agent of MSX disease, and *Perkinsus marinus*, the causative agent of Dermo disease in eastern oysters, has led to a dramatic decrease in landing weight along the East coast (Andrews 1988, Haskin and Andrews 1988) and in the Gulf of Mexico of the United States (Andrew and Ray 1988).

Eradication of pathogens, drug treatments, vaccination, and breeding for genetic improvement are strategies usually used to control infectious diseases in animals, however, most of these approaches are not practical in oysters. Eradication of pathogens in oysters is difficult because they inhabit open bays and estuaries. Use of chemotheraputants is only feasible for broodstock and larvae in hatcheries, and the concern of inducing antibiotic-resistant pathogens limits the practice (Elston 1984, Calvo and Burreson 1994, Faisal et al. 1999). Moreover, vaccination cannot be used due to the lack of an acquired immunity in oysters (Ratcliffe et al. 1985). Therefore, producing disease resistant strains by genetic improvement may be a feasible strategy to control oyster infectious diseases.

Unfortunately, results of conventional genetic techniques, such as chromosome manipulation and selective breeding are disappointing because disease resistance related genes are difficult to enhance or select specifically. For example, by chromosome manipulation, triploid oysters were generated and found to be as susceptible to Dermo as diploid oysters (Barber and Mann 1991). By selective breeding of spontaneously

occurring genetic resistant strains to MSX, a resistant strain was developed (Ford and Haskin 1987), but undesirable genetic traits were found such as slow growth and lack of resistance to Dermo (Burreson 1991). Currently, the development of gene transfer techniques allows disease-resistant genes such as antimicrobial peptides to be specifically introduced into oysters to enhance disease resistance.

Because gene expression is mainly controlled by the promoter, selection of a promoter to control gene expression is a critical step for studying disease resistance in oysters. Promoters originating from the same species (homologous promoters) allow better control of gene expression than those from different species (heterologous promoters)(Du et al. 1992, Takagi et al. 1994, Devlin et al. 1994). When homologous promoters are not available, heterologous promoters can be adapted, based on the phylogenetic conservation of gene regulation.

Promoters can be categorized into one of the two categories: constitutive or inducible promoters. Constitutive promoters constantly drive gene expression and are used to express genes continuously. Inducible promoters respond to specific inducers to drive gene expression and can be used to regulate transgene expression. Specific inducers can be: 1) physical factors such as radiation (Datta et al. 1992, Hallahan et al. 1995), heat shock (Lis et al. 1983, Bienz and Pelham 1982, Zhang et al. 1998), and light (Peng et al. 1998); 2) chemical factors such as ethanol (Umeno et al. 1988), hormones (White 1997), or metals (Murphy et al. 1990, Inoue et al. 1992, Kinoshita et al. 1994); or 3) biological factors such as viruses (Hug et al. 1988, Chang et al. 1991).

Inducible promoters are the best candidates for controlling the expression of disease-resistance (effector molecule) genes. Inducible gene expression patterns can

prevent host tissues from being damaged by over-expression of effector molecules (Babior 1984). For oysters, because no homologous inducible promoter has been cloned, the adaptation of an inducible promoter from a phylogenetically related species becomes necessary. Thus, the promoter of the snail, *Biomphalaria glabrata*, heat shock protein 70 (HSP 70) gene and the promoter of the moth, *Hyalophora cecropia*, cecropin B gene were chosen for evaluation.

The regulation of heat shock response genes, including the heat shock DNA sequence in the promoter and heat shock transcription factors of the host, are conserved among species (Holmgren et al. 1981, Pelham 1982, Corces et al. 1981, Scharf et al., 1990). For example, the heat shock 70 promoter from *Drosophila* was shown to function in a heat inducible manner in cell lines derived from a broad spectrum of animal classifications, such as sea urchin (echinoderm)(McMahon et al. 1984), oyster (mollusc) (Boulo et al. 1996), mosquito (insect)(Zhao and Eggleston 1999, Shotkoski et al. 1996, Miller et al. 1987), frog (amphibian)(Voellmy and Rungger 1982), trout (fish)(Inoue et al. 1990), and mammalian cells (Roigas et al. 1997).

In addition, heat shock response genes can be induced by physiological stresses including trauma, inflammation, infection, and cancer (Morimoto and Santoro 1998), chemical stress such as ethanol (Hahn et al. 1985), and physical stress such as placement in a magnetic field (Goodman and Blank 1998). Therefore, heat shock promoters have the potential to drive expression of effector molecule genes in response to stressors. For this reason, we looked first at the promoter from snail (*Biomphalaria glabrata*) heat shock 70 gene (SHSP-70)(Yoshino et al. 1998).

The cecropin B promoter, which functions in a similar way to promoters of mammalian acute phase response (APR) genes, can be induced by pathogen invasion early in the infection and shut down after the pathogens have been eliminated (Boman et al. 1991, Baumann and Gauldie 1994). The APR genes and their regulatory components, including cell receptors, signal transduction pathways, transcription factors and promoter sequences (Hultmark 1993), are conserved. For example, the binding site for nucleus factor kappa-B (NF-κB), which regulates most APR genes, is present in the cecropin B promoter and all other lytic peptide promoters that have been cloned to date (Barra et al. 1998).

Although the cytokines that mediate APR have not been identified in oysters, cytokine-like molecules of interlukin (IL)-1, IL-6, tumor necrosis factor-alpha, which mediate the regulation of APR genes in mammals (Heinrich et al. 1990, Baumann and Gauldie 1990, Castell et al. 1989) and their receptors were found in blue mussels, *Mytilus edulis*, (Hughes et al. 1990, 1991, 1992, Ottaviani et al. 1993, 1997). The presence of cytokine-like molecules and their receptors in a bivalve suggests that they might exist in oysters to mediate the signal for the cecropin promoter. Thus, the cecropin B (APR) promoter was chosen for evaluation.

To conduct *in vitro* gene function studies, a well-established cell culture and gene expression system is needed but this is not available for oysters. Thus, the optimized cell culture conditions from our previous studies were used to establish gene transfer and expression systems.

The objectives were: 1) optimization of transfection conditions for oyster cells, 2) evaluation of SHSP-70 promoter, and 3) evaluation of APR promoter.

Materials and Methods

Plasmids

Plasmid (pPC) containing the SHSP-70 promoter with a luciferase reporter gene, was provided by Dr. Yoshino (Department of Pathobiological Sciences, University of Wisconsin at Madison). The snail heat shock promoter was deleted to generate plasmid (pNPC) as a negative control for the transfection assay. Plasmid (pAPR/GL3E) containing the APR promoter was constructed by PCR amplification of the cecropin B promoter from the cecropin B gene followed by cloning into the multiple cloning site of pGL3E in frame with the luciferase gene (Appendix F).

Cell Culture

Oyster ventricle cells were dissociated using pronase as described in Chapter 2. The dissociated cells were resuspended in LA-3 medium (Appendix D) and cultured in 96 well-plates at a density of 4×10^5 cell/100 µl/well at 25° C for 3 d to allow cell attachment and spreading.

Cell Transfection and Heat Shock

Transfection was performed using an Effectene transfecting kit (Qiagen Inc. Valencia, California) containing EC buffer for DNA dilution, Enhancer for condensing DNA and Effectene for the formation of lipid-DNA complex to deliver DNA into cells. For the DNA to be condensed by the Enhancer, the concentration of DNA specified for each study was diluted to 30 µl using EC buffer followed by the addition of Enhancer at ratio of 1:8 (µg DNA:µl Enhancer) and incubation at room temperature for 5 min. Second, for the formation of Effectene-Enhancer-DNA complexes, Effectene was diluted to 20 µl using EC buffer and added to the condensed DNA. Then, the Effectene-

Enhancer-DNA complexes were added to wells containing cells after incubation at room temperature for 10 min. Based on preliminary studies, cells were incubated with Effectene-Enhancer-DNA complexes at 25° C for 16 h and were heat shocked at 40° C for 1 h followed by recovery at 25° C for 12 h before the luciferase assay.

Luciferase Assay

Cell extract preparation: Luciferase activity of cells was measured using a Luciferase assay system (Promega Corporation, Madison, Wisconsin). The medium was removed and the cells were washed once using 100 µl of sterile artificial seawater (FASW) (Instant Ocean, Aquarium Systems Inc., Mentor, Ohio). Fifty µl of cell culture lysis reagent (25 mM Tris-phosphate pH 7.8; 2 mM DTT; 2 mM 1,2-diaminocyclohexane -N,N,N',N'-tetraacetic acids; 10% glycerol; 1% Triton® X-100) were added to lyse cells. Cells attached to the bottom of wells were removed by pipetting the cell culture lysis reagent into the wells. Cell lysates were transferred to a 0.2 ml tube, incubated 5 min at room temperature, and centrifuged at 12,000 x g for 5 sec. Supernatants were transferred to a new micro tube and stored at – 80°C until luciferase activity could be measured.

Measurement of luciferase activity: Cell extracts and the assay reagents were thawed in a 25° C water bath. Assays were conducted at room temperature (25° C). Twenty µl of cell extract were added to a 1.8-ml glass vial (Fisher Scientific, Pittsburgh, Pennsylvania) and placed in the chamber of a luminometer (Model 20e, Turner Designs, Sunnyvale, California). Then, 100 µl of luciferase assay reagent containing luciferase substrates were injected into the vial. Light production was measured for 10 sec. The results were recorded as relative light units (RLU).

Study 1: Optimization of DNA to Effectene Ratio

Cells receiving no Effectene or DNA and cells receiving Effectene only were used as negative controls. Three pPC DNA concentrations (0.1, 0.2, and 0.3 μg) at three ratios (1:10, 1:25 and 1:50) of DNA to Effectene were evaluated. The optimal ratio of DNA to Effectene was chosen for the following studies.

Study 2: Optimization of DNA Concentration

Cells were transfected with 0.2, 0.3 and 0.4 μg of pPC DNA at a ratio of 1:10 of DNA to Effectene for optimization of DNA concentration. Cells receiving Effectene only (0 μg DNA) were used as a negative control. The optimal concentration was chosen for the following studies.

Study 3: Heat induction of SHSP-70 Promoter

Cells were transfected with 0.3 μg of pNPC or pPC DNA to determine whether heat induction of luciferase activity could be mediated by the SHSP-70 promoter. Cells without Effectene and cells with Effectene only were used as negative controls. To decrease the variation per treatment, 12 replicate wells of each cell treatment were prepared. Each of 12 wells was lysed with 20 μl of cell culture lysis buffer and every 4 wells were pooled which resulted in each treatment containing 3 replicate pools. The following studies were all conducted as described above.

Study 4: Time course response of SHSP-70 Promoter

Cells were transfected with 0.3 μg pPC DNA to determine a time course response. Luciferase activity was measured at 0, 3, 6, 9,12, and 24 h after heat shock. The time yielding the highest expression was chosen for the following studies.

Study 5: Temperature Course Response of Snail Heat Hhock 70 Promoter

Cells were transfected with 0.3 μg pPC DNA to evaluate the temperature yielding the best luciferase activity. Transfected cells were heat shocked at 30, 35, 40, or 45° C for 1 h. Luciferase activities were measured 12 h after heat shock. The optimal heat shock temperature was chosen for the following study.

Study 6: Evaluation of the APR Promoter

Cells transfected with pPC were heat shocked to serve as a positive control of transfection. Cells transfected with pPC but not shocked, were used to provide a negative control for activation of the promotor. All transfections in this study used a ratio of 1:10 (DNA to Effectene) and 0.3 μg DNA. Cells transfected with pGL3E were used as a negative (no APR promoter) control while cells transfected with pAPR/GL3E were used for the evaluation of induction of the APR promoter sequence. Ten μg/ml/well of LPS which is prepared form *Salmonella enteritidis* using phenolic extraction and gel filtration chromatography (Sigma, product number L 7770) and/or 20 pg/ml/well of recombinant mouse IL-1-alpha (Sigma, product number I 5396) were added to induce the APR promoter 16 h after transfection. Luciferase activity was measured at 6 h and 12 h after the addition of LPS and IL-1.

Data Analysis

Each treatment in each Study contained 3 replicates. Studies 1 to 5 were repeated twice and Study 6 was conducted once. Data from each study were analyzed using analysis of variance (ANOVA). Duncan's multiple range test was performed when significant differences were found ($P < 0.05$).

Results

Study 1: Optimization of DNA to Effectene Ratio

Significant differences in luciferase activity were found among cells transfected at a ratio of 1:10 of DNA to Effectene compared to those of 1:25 and 1:50. Cells transfected with 0.3 μg DNA showed the highest (0.069 ± 0.020) ($P < 0.05$) luciferase activity, and were significantly different compared to the cells of DNA-only control (0.006 ± 0.020) and Effectene only control (0.007 ± 0.025) (Figure 4-1). Cells transfected at ratio of 1:25 and 1:50 showed no significant difference compared to control cells and cells transfected with DNA. Therefore, a ratio of 1:10 was chosen for further studies.

Study 2: Optimization of DNA Concentration

Cells transfected with 0.2, 0.3 μg DNA showed a significant difference in luciferase activity compared to cells receiving Effectene only (0.010 ± 0.015) (Figure 4-2). Cells transfected with 0.3 μg DNA showed the least variation and the highest luciferase activity (0.108 ± 0.062) compared to other treatments. Thus, 0.3 μg of DNA was chosen for further studies.

Study 3: Heat Induction of SHSP-70 Promoter

Heat induction of luciferase activity was seen only in cells containing the SHSP-70 promoter. Cells transfected with pNPC showed no significant increase in luciferase activity with or without heat shock (Figure 4-3). A ten-fold increase in luciferase activity was found after heat shock, between cells transfected with pPC and cells transfected with pNPC.

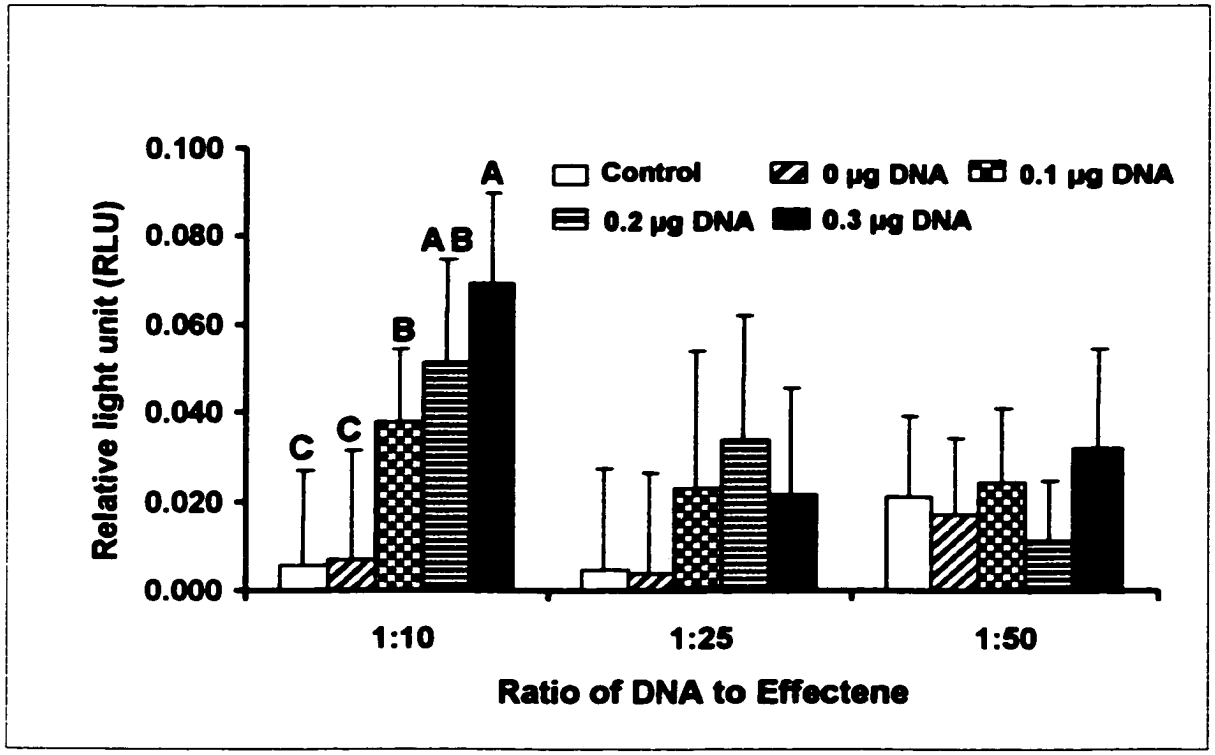

Figure 4-1. Optimizing the of DNA to Effectene ratio. Cells were transfected with Effectene at the indicated DNA concentration and DNA to Effectene ratio. After transfection, cells were heat shocked at 40° C for 1 h, and recovered at 25° C for 12 h and used in a luciferase assay. Bars without letters indicate no significant difference in luciferase activity compared to controls and Effectene only cells. Bars sharing letters (A, B, and C) were not significantly different (n = 6, $P > 0.05$).

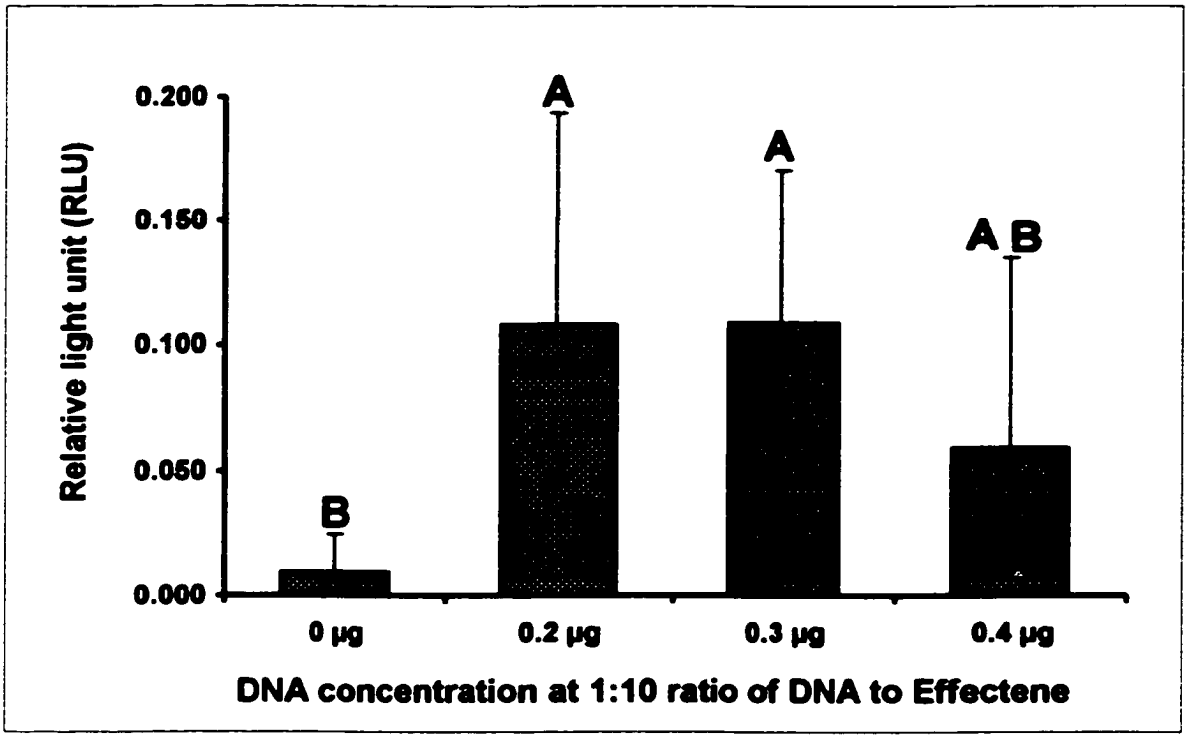

Figure 4-2. Optimization of DNA concentration. Cells were transfected with the indicated DNA concentration. Luciferase activity was measured 12 h after heat shock at 40° C for 1 h. Bars sharing letters (A and B) were not significantly different (n = 6, $P >$ 0.05).

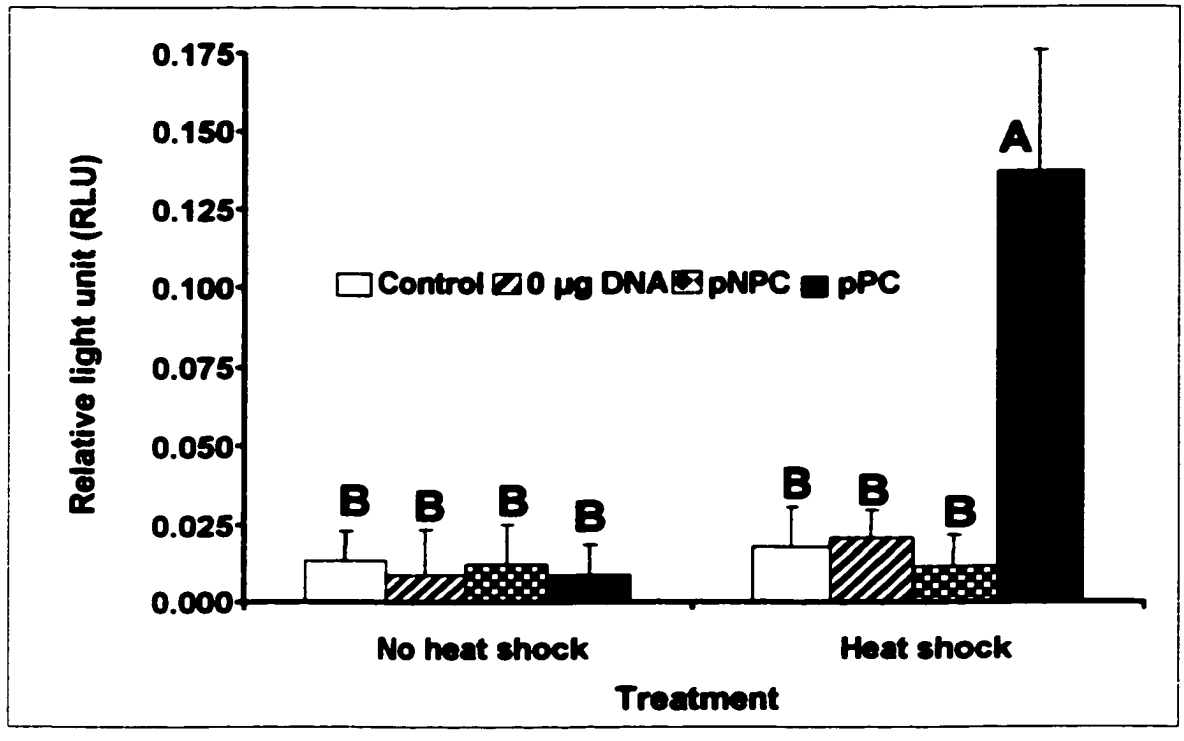

Figure 4-3. Heat induction of the heat shock protein 70 promoter from snail (SHSP-70). Cells were transfected with pNPC containing no snail heat shock promoter and pPC containing the SHSP-70 promoter. Bars sharing letters (A and B) were not significantly different (n = 6, $P > 0.05$).

Study 4: Time Course Response of SHSP-70 Promoter

A significantly higher luciferase activity (0.114 ± 0.033) was detected at 12 h, as opposed to the other time points (Figure 4-4). A significant increase in luciferase activity was not detected until 6 h (0.051 ± 0.014) while a significant decrease of luciferase activity was found at 24 h (0.080 ± 0.018)(Figure 4-4). Therefore, 12 h was chosen for the following studies.

Study 5: Temperature Effect on Inducing the SHSP-70 Promoter

The optimal heat shock temperature was 40 °C. Cells receiving no DNA, and cells transfected with 0.3 μg pPC DNA showed no significant difference in luciferase activity after heat shock at 30° C (0.001 ± 0.007 vs. 0.006 ± 0.010). A significantly higher luciferase activity was found at 40° C (0.102 ± 0.030) followed by 45° C (0.084 ± 0.011) and 35° C (0.050 ± 0.027)(Figure 4-5).

Study 6: Evaluation of APR Promoter

The induction of the APR promoter was not significantly different from the controls at 6 h and 12 h after the addition of LPS and IL-1. The heat-shock control cells transfected with pPC showed a significantly higher luciferase activity compared to those without heat shock (Figure 4-6). The pAPR/GL3E transfected cells showed higher luciferase activities than those of pGL3E transfected cells at 6 h and 12 h after the addition of IL-1 (Figure 4-6) but the difference was not significant ($P > 0.05$).

Discussion

A system for gene transfer and expression is essential for *in vitro* studies. *In vitro* gene transfer is well established for vertebrate cells but *in vitro* gene transfer for invertebrates is limited to a few species such as *Drosophila*, mosquito, and snail

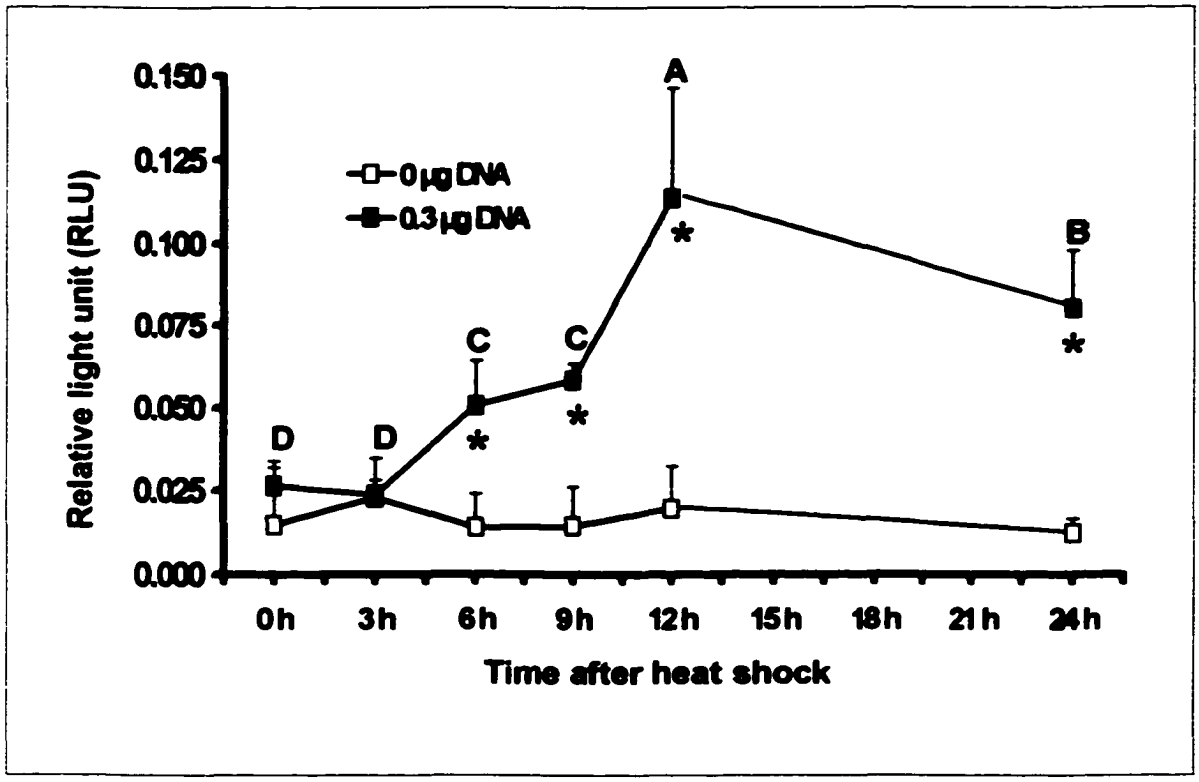

Figure 4-4. Activation time course of the snail heat shock protein 70 promoter gene. Cells were transfected with pPC DNA and heat shocked at 40° C for 1 h followed by a luciferase assay at the indicated time. * indicates a significant difference in luciferase activity was found between cells transfected with 0 µg DNA and 0.3 µg DNA. Points of cells transfected with 0.3 µg DNA sharing letters (A, B, C, and D) were not significantly different (n = 6, $P > 0.05$).

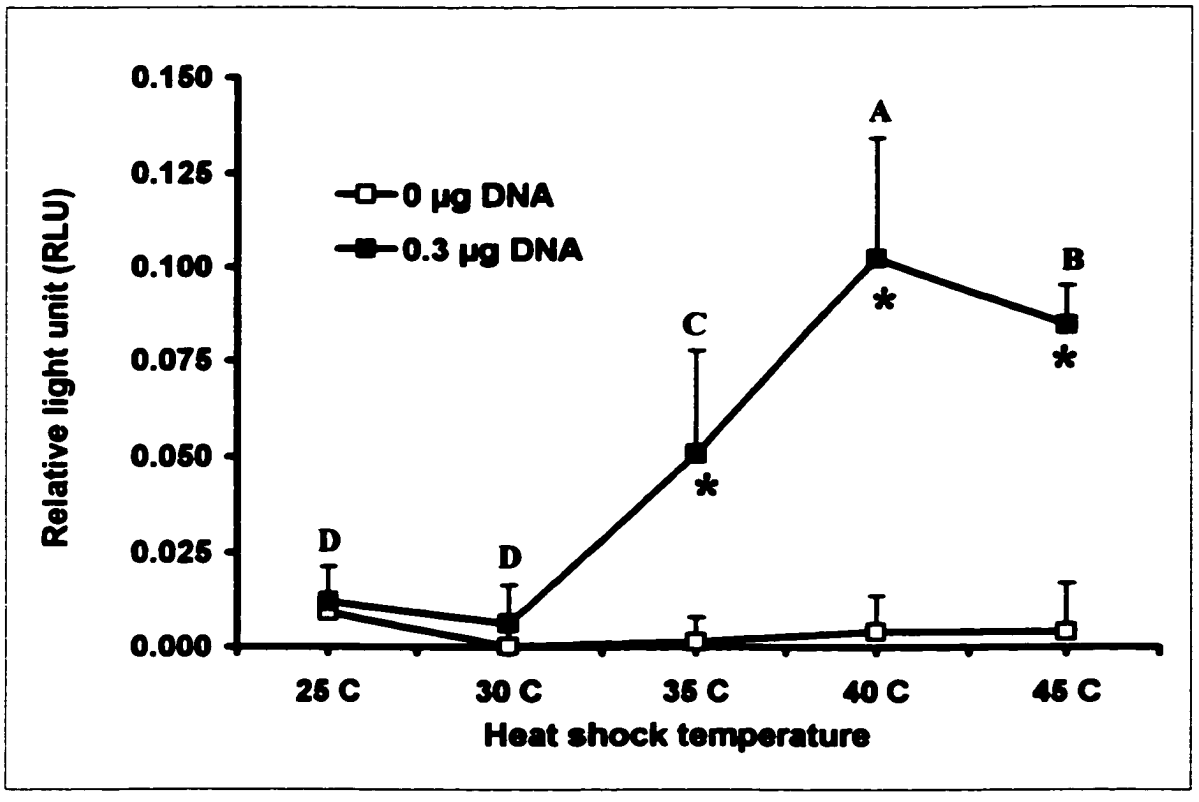

Figure 4-5. Temperature optimization for heat shock induction. Cells were transfected with pPC DNA and heat shocked at the indicated temperature for 1 h. Luciferase assays were conducted 12 h after heat shock. * Indicates a significant difference in luciferase activity was found between cells transfected with 0 µg DNA and 0.3 µg DNA. Points of cells transfected with 0.3 µg DNA sharing letters (A, B, C, and D) were not significantly different ($n = 6$, $P > 0.05$).

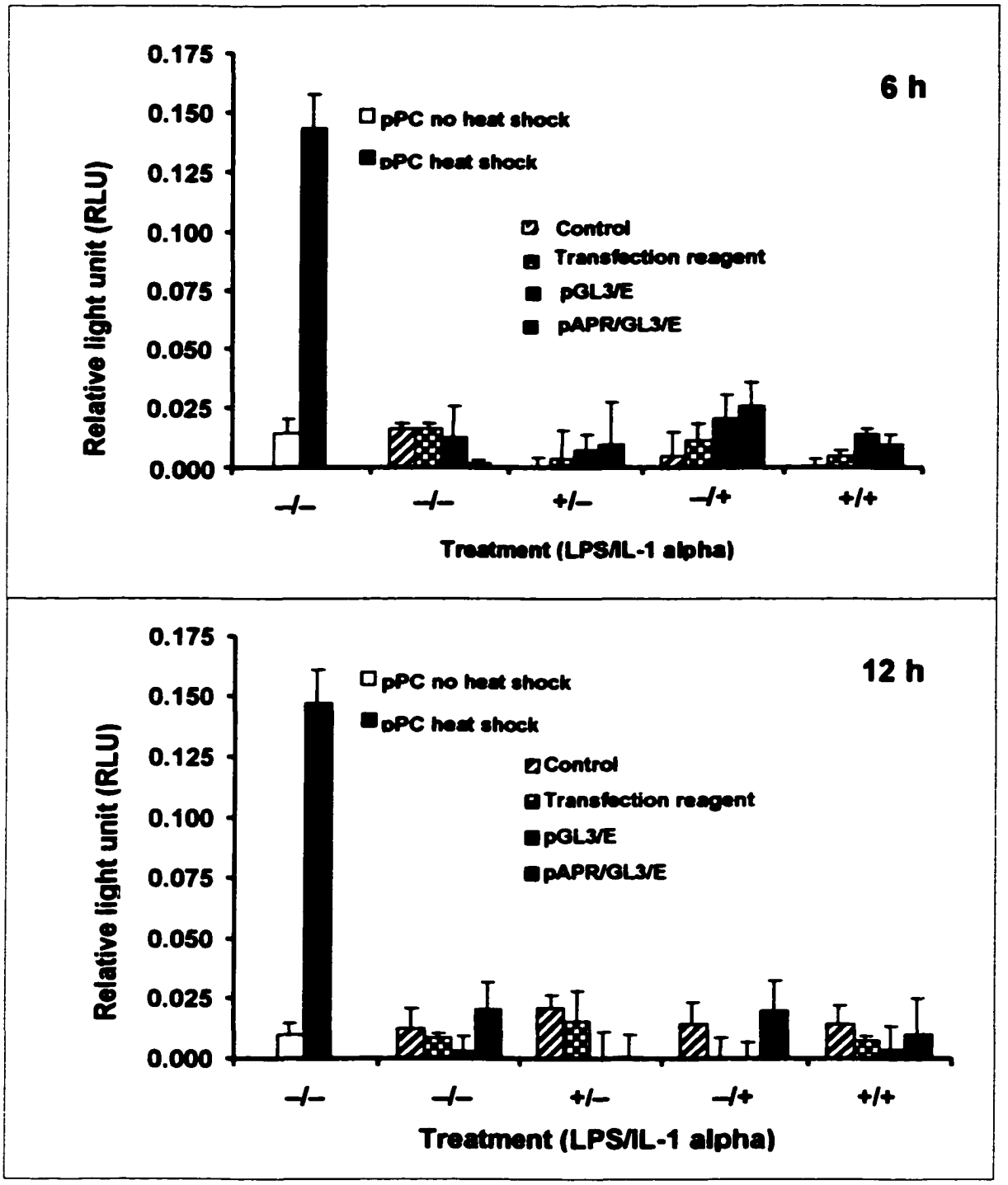

Figure 4-6. Induction of cecropin B (APR) gene promoter. Cells were transfected with pPC DNA as transfection positive controls. The pGL3/E and pAPR/GL3/E transfected cells were used to evaluate the induction of the APR promoter. LPS and mouse IL-1 alpha were added 16 h after transfection. Luciferase activity was measured at 6 h and 12 h after adding LPS and IL-1 alpha ($n = 3$, $P < 0.05$).

(Marshall 1998, Lardans et al. 1996). No cell line is available and the lack of established primary cell culture conditions hinder the development of a gene expression system for marine bivalves. In this study, we developed the first reported serum-free gene expression system used for evaluating promoters.

A primary heart cell culture from the pacific oyster (*C. gigas*) is the only marine bivalve somatic cell type, which has been transfected (Boulo et al. 1996). However, the cells were transfected with a lipid-based transfection reagent N-[1-(2,3-dioleoyloxy)-N,N,N-trimethylammonium methylsufate] (DOTAP)(Roche, Mannheim, Germany) and the cells were cultured in 2X L-15 medium containing 10% fetal bovine serum (FBS). The DOTAP was one of the first lipid-based transfection reagents and at least 5 to 10 times amount of DNA was required to transfect cells compared to more recent transfection reagents. Although DOTAP is often used to mediate gene delivery for insect cells (Lardans et al. 1996, Zhao and Eggleston 1999), it is not a universal transfection reagent that can transfect every cell type. To identify a more efficient transfection reagent, preliminary studies of comparing DOTAP, Lipofectamine (Life Technologies, Gaithersburg, Maryland), and Effectene were conducted. Ventricle cells could not be transfected with either DOTAP or Lipofectamine, while ventricle cells could be transfected by Effectene. In addition, the use of Effectene required 10 times less DNA than those of using a DOTAP transfection reagent.

Using L-15 medium, which is designed for mammalian cell culture, is not optimal for oyster cell primary cultures. Meanwhile, the addition of FBS containing unknown components (Freshney 1994), increases the complexity and decreases the usefulness of *in vitro* assays. Purification of recombinant antimicrobial peptides (Choi 1996) and studies

of *P. marinus* proteases (La Peyre et al. unpublished data) were hindered by the presence of serum in culture medium. In this study, LA-3 medium was used. This is a serum free, chemically defined media that has been optimized for oyster cells (La Peyre et al. unpublished data). Results of this study indicated that LA-3 medium could support ventricle cells to allow gene expression studies.

The study of heat shock protein of oyster hemocytes indicated that an increase in heat shock proteins was found as early as 1 h after heat shock at 41° C (Tirard et al. 1995), but in this study, a significant increase of luciferase activity was not found 3 h after heat shock at 40° C. Whether the heat shock temperature affect the time needed for the detection of significant increase of luciferase remains to be studied.

While promoter sequences of heat shock genes are conserved among different species, the temperature required to induce the heat shock promoter is dependent on the physiological temperature of the individual host species (Lindquist 1986, Abravaya et al. 1991). Induction of *Drosophila* heat shock 70 promoter is found with a temperature shift from 25° C to above 28° C in *Drosophila*. However, in human H9T cells, the *Drosophila* HSP 70 promoter does not respond to 37° C which is the normal culture temperature of H9T cells, but does respond to a shift from 37° C to 42° C, which activates human heat shock promoters (Schweinfest et al. 1988). These results indicate that the activation of heterologous heat shock promoters is determined by host transcription factors which can be activated by heat stress or other stresses such as chemical stress, i.e. cadmium chloride (Liu et al. 1994).

In the study of heat shock proteins of oyster hemocytes, the heat shock proteins were induced by a shift from 20° C to above 40° C (Tirard et al. 1995) and a shift from

12° C to 37° C (Clegg et al. 1998.), suggesting that heat shock transcription factors are activated at 37° C and above. In this study, a shift from 25° C (cell culture temperature) to 35° C and above could induce the SHSP-70 promoter, implying that heat shock transcription factors recognize the SHSP-70 promoter and are activated at 35° C.

Induction of the SHSP 70 promoter was not seen in the serum-free oyster cell cultures of this study, either at normal culture temperatures (25° C) or after heat shock (30° C). The induction of the *Drosophila* HSP 70 promoter was found at 18° C constant culture temperature (Boulo et al. 1996), without heat shock treatment. The stresses of cell culture conditions, such as the presence of serum, which can increase the basal transcription of HSP 70 genes (Wu et al. 1985) or the transfection process itself, could be causing this induction.

The results of our study suggest that the heat induction of SHSP-70 promoter is tightly regulated by the intensity of heat applied. This heat inducible serum-free gene expression system will benefit oyster *in vitro* studies, such as that of lytic peptide gene function or the function of other genes providing disease resistance.

Activation of a cecropin gene by LPS is an immediate response in hemocytes of channel catfish and *Drosophila*, and in *Drosophila* embryonic cells. In *Drosophila* cell lines, the time required for activation of a cecropin gene after the addition of LPS (0.1 ng/ml to 100 μg/ml) was 2 h for a malignant blood neoplasm-2 (mbn-2) cell line (Samakovlis et al. 1992) and 4 h for an embryonic SL2 cell line (Samakovlis et al. 1990). The number of mbn-2 cells responding to LPS by cecropin expression was 10 fold higher than that of SL2. For channel catfish leukocytes transfected with a cecropin B promoter linked to a gene for green fluorescent protein (GFP), the induction of a two-fold increase

of GFP mRNA was detected 10 h after addition of 125 μg/ml LPS (Zhang et al. 1998). Although primary ventricle cells cultured in this study contained at least 30 to 40% hemocytes, the induction of the cecropin B promoter in our studies was not seen at 6 h and 12 h after the addition of LPS.

The absence of serum may be a valid explanation for the lack of cecropin B promotor induction. All the media used for lytic peptide gene expression assays in insects, fish or other animals, contain serum. The addition of serum is mainly for cell growth support but the components of serum may be required as cofactors to stimulate hemocyte cytokine production thereby inducing the APR promoter. One of the key serum factors necessary for macrophages to recognize LPS is LPS-binding protein (Mathison et al. 1992). In mammals, the NF-kB transcription factor, a major coordinator of innate immunity genes (Siebenlist et al. 1994), is activated by LPS or pathogen challenge to bind the promoter NF-kB sequence and facilitate the activation of innate immunity genes. In insects, similar transcription factors are involved in the same role in the activation of lytic peptide genes (Ip et al. 1993, and Petersen et al. 1995). Whether LPS binding protein is required for activation of the transcription factors in mammals or insects is unknown. However, LPS-binding protein can increase the production of cytokines such as tumor necrosis factor (TNF) in LPS-treated macrophages (Mathison et al. 1992) and the production of those cytokines can enhance the expression of effector molecule genes. It remains to be studied whether LPS binding protein or other serum factors are required for ventricle cells to produce cytokines or activate the cecropin promoter.

The most probable cause of the unresponsive APR promoter after the addition of IL-1 is the dose and specificity of IL-1. The dose of exogenous IL-1 might be not high enough to activate APR promoters. In the snail hemocyte phagocytosis assay, 500 ng/ml IL-1-alpha, which is 25-fold more than the IL-1-alpha concentration in this study, was used to enhance phagocytosis activity (Ottaviani and Franchini 1995). Meanwhile, cytokines may show species specificity for mollusc hemocytes. In the hemocyte migration assay of two fresh water snails, TNF-alpha (! U/ml) enhanced the migration of hemocytes of *Planorbarius corneus* but not those of *Viviparus ater* (Ottaviani and Franchini 1995).

The lack of serum factors, and the low dose and specificity of IL-1 might explain why the APR promoter was not activated when both LPS and IL-1 were added. Further studies need to be conducted to verify the above possible explanations individually.

In conclusion, an *in vitro* gene transfer, heat inducible, serum-free gene expression system was established. By using this system, we found that the SHSP-70 promoter was activated in response to heat shock but the promoter (APR) of the cecropin B gene was not activated after the addition of IL-1 and LPS. Further *in vitro* and *in vivo* studies are needed to evaluate APR promoter function. The establishment of a transfection system of ventricle cells, which are cultured in serum-free medium can be used to study gene regulation and function without the interference of serum.

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CHAPTER 5 EVALUATION OF THE EFFECTS OF ANTIMICROBIAL PEPTIDE GENE TRANSFER ON THE IMMUNITY OF THE EASTERN OYSTER

Introduction

The value of oyster production exceeded 100 million dollars in United States in 1995 (MacKenzie 1996). However, in addition to overharvesting and water quality deterioration, diseases of oysters and contamination with human pathogens are two major factors hampering further development of the oyster industry. Diseases that affect oysters have caused a decrease in oyster harvests. Pathogens causing oyster diseases include viruses (Farley et al. 1972, 1976), bacteria (Tubiash et al. 1973, Elston et al. 1982, Dungan et al. 1989) and protozoa (Andrews 1967). The protozoan *Perkinsus marinus* is recognized as a major pathogen responsible for a decrease in production of the eastern oyster in the Gulf of Mexico (Andrews and Ray 1988).

Human pathogen contamination of oysters has resulted in decreased consumer acceptance and a decrease in the market price (Hargis and Haven 1988). Oysters are cultured along the coast and estuarine areas where algae and bacteria are abundant. They filter surrounding water at a rate of up to 34 liters per hour to obtain algae as food (Galtsoff 1964). Unfortunately, bacteria also accumulate in oysters during feeding. The accumulated bacteria include human pathogens such as *Vibrio vulnificus*, which is non-pathogenic to adult oysters but can cause gastroenteritis and septicemia in humans. Between 1988 and 1996, a total of 422 cases of *V. vulnificus* infection were reported (Shapiro et al. 1998). Forty-one percent of the patients had consumed raw oysters. Sixty- one percent of the patients who consumed raw oysters died as a result of the infection (Shapiro et al. 1998). Therefore, contamination with human pathogens becomes

a major concern in summer months, when the pathogens are most abundant (Wright et al. 1996). To alleviate contamination problems, radiation and depuration (culturing oysters in clean water) have been suggested to reduce bacteria loading in oysters. However, radiation did not receive Food and Drug administration (FDA) approval, and depuration was not efficient in eliminating or reducing human pathogens such as *V. vulnificus* (Tamplin and Capers 1992, Jones et al. 1991). Although, adding food preservatives such as 0.05% diacetyl (2,3-butadione) can also eliminate *V. vulnificus*, the cost and lack of consumer acceptance have limited the application of this practice (Sun and Oliver 1994).

Vaccine immunization is not possible for oysters, since they lack amnesis and cannot mount a response based on pathogen recognition. Because of this fact, the word "immunity" must be understood in a very broad sense when applied to oysters, as it is here. One feasible way to solve disease and contamination problems is to enhance non-specific oyster immunity. Oysters rely on innate immunity including cellular immunity such as phagocytosis of hemocytes, and humoral factors such as lectins, agglutinins, protease inhibitors, lysozymes and antimicrobial peptides (Ford and Tripp 1996). Improvement in the production of these endogenous molecules or the addition of a functional immunity factor (effector molecule) such as an antimicrobial peptide gene to the oyster genome may increase disease resistance and reduce accumulation of human pathogens.

Antimicrobial peptides were first isolated from insects (Boman et al. 1972). Thereafter, antimicrobial peptides have been found in plants (Broekaert et al. 1997), and animals (Boman 1995) as non-specific immune effector molecules. These antimicrobial peptides typically consist of 23 to 39 amino acids, and can be either stored

(compartmentalized) in cells such as macrophages for intracellular killing, or secreted for extracellular killing of pathogens (Boman 1995). In addition to antibacterial, antifungal, and antiparasitic activities (Boman 1991, 1995), these antimicrobial peptides confer diverse functions, such as binding to bacterial endotoxin (Sawyer et al. 1988), promoting wound healing (Murphy et al. 1993), stimulating monocyte migration (chemotaxis) to targeted pathogens (Territo et al. 1989), regulating cell response to cytokines (Mattsby-Baltzer et al. 1996), and enhancing the growth of mouse fibroblasts (Reed et al. 1992).

Antimicrobial peptides are found in many species that are important to aquaculture such as loach, *Misgurnus anguillacaudatus* (Park et al. 1997), catfish, *Parasilurus asotus* (Park et al. 1998), flounder, *Pleuronectes americanus* (Cole et al. 1997), horseshoe crab, *Tachypleus tridentatus* (Nakamura et al. 1988), blue crab, *Callinectes sapidus* (Khoo et al. 1999), shrimp, *Panaeus vannamei* (Destoumieux et al. 1997), sea hare, *Aplysia kurodai* (Takamatsu et al. 1995), mussels, *Mytilus edulis* (Charlet et al. 1996), and oysters, *Ostrea edulis* and *Crassostrea gigas* (Hubert et al. 1996). *In vitro* research has been conducted to evaluate antimicrobial activity on oyster and human pathogens. For example, the parasitic oyster protozoa, *Bonamia ostreae* and *P. marinus*, and various species of *Vibrio* bacteria were effectively killed by tachyplesin 1 isolated from the hemocytes of the Japanese horseshoe crab *Tachypleus tridentatus* (Morvan et al. 1997). Furthermore, an analog of polyphemusin, an antimicrobial peptide derived from the hemocytes of the North American horseshoe crab *Limulus polyphemus*, effectively inhibited the growth of *P. marinus*, and human pathogens (Pierce et al. 1997).

Although the source and the amino acid sequences of antimicrobial peptides vary, the net positive charge (cationic) and amphipathic alpha helix or beta sheet structure that

contains mostly hydrophobic amino acids on one side and hydrophilic amino acids on the other, are characteristics common to most antimicrobial peptides (Segrest et al. 1990, Saberwal et al. 1994, Maloy and Kari 1995, Boman 1995). The positive charge and amphipathic structures of antimicrobial peptides are correlated with their antimicrobial activities (Lee et al. 1986, Oh et al. 1999). In keeping with this basic structure, antimicrobial peptide analogs (Maloy and Kari 1995, Saido-Sakanaka et al. 1999), newly designed antimicrobial peptides (Blondelle and Houghten 1992, Bessalle et al. 1993, Javadpour et al. 1996, Oren et al. 1997, Tossi et al. 1997) and hybrid peptides (Shin et al. 1998, 1999) were developed. Antimicrobial peptides are promising candidates for enhancing oyster innate immunity due to their broad-spectrum antimicrobial activities, multiple functions, and availability of various analogs.

The antimicrobial peptide cecropin B has been studied extensively. Cecropin B was isolated from the giant silk moth *Hyalophra cecropia* (Hultmark et al. 1980) and possesses antimicrobial (Hultmark et al. 1982) and anticancer activities (Moore 1994). Channel catfish, *Ictalurus punctatus* showed enhanced resistance to the bacterial pathogen *Edwardsiella ictaluri* after *in vivo* administration of cecropin B and analog peptides (Kelly et al. 1993). Moreover, transfer of the cecropin B gene to channel catfish reduced bacteria loading and mortality after challenge with *E. ictaluri* (Cooper et al. unpublished data). Because of promising preliminary results and availability, the gene of cecropin B peptide was chosen for *in vivo* oyster studies. In addition, the gene of a newly designed 24 amino acid peptide (LOKI-24) with amphipathic structure was chosen for evaluation.

Gene transcription can be detected at the RNA level by Northern blot (Alwine et al. 1977) and RNase protection assay (Belin 1996). However, reverse transcriptase-polymerase chain reaction (RT-PCR)(Kawasaki 1990) has increasingly been used as an alternative due to better sensitivity. The first step in the procedure is to isolate RNA from experimental cells and transcribe it back into single strand complementary DNA (sscDNA) using a reverse transcriptase. The sscDNA can be used in a polymerase chain reaction (PCR) for amplification. Often, the beta actin gene, which is constitutively expressed in most cell types, is used either as an internal control to monitor reverse transcription or as a reference to quantify target gene expression (Kinoshita et al. 1994).

One drawback to this method however, is that genomic DNA (gDNA) contamination is inevitable during RNA isolation. The gDNA is co-amplified with sscDNA when PCR is performed, and can lead to false positive results of RT-PCR. This problem can be solved using PCR primers spanning across introns of the actin gene to generate PCR products, which differ in size from the shorter cDNA product. To date, only one partial sequence of actin cDNA from the gills of eastern oyster has been reported (Unger and Roesijadi 1993). Furthermore, whether or not the actin gene contains an intron is unknown. In order to use the gill actin gene as an internal control in hemocyte RT-PCR, the first objective of this study was to determine whether the gill actin gene contains an intron and expresses in hemocytes. The second objective was to evaluate the feasibility of the transfer of antimicrobial peptide genes controlled by a cecropin B promoter to enhance oyster immunity. Because hemocytes are the defense cells in oysters, the hemocyte number and hemocyte killing ability (index) were measured. In addition, bacterial hemolymph loading, bacterial tissue loading and *P.*

marinus tissue loading measurements were measured as indices for immunity enhancement.

Materials and Methods

Oysters

Eastern oysters were collected at Grand Isle, Louisiana, and were maintained in a recirculating system with ultraviolet-treated artificial seawater (Instant Ocean, Aquarium Systems Inc., Mentor, Ohio) at 15‰ salinity and 25° C at the Department of Veterinary Science, Louisiana State University Agricultural Center.

Hemolymph Collection

Oysters were notched at a point 2/3 of the distance from the hinge along the shell edge of the dorsal lateral side. This site is near the adductor muscle sinus and is convenient for collecting hemolymph or for plasmid injection. Hemolymph was collected from the adductor muscle sinus using a 3-ml syringe and a 25-G needle. To avoid shearing hemocytes, hemolymph was slowly collected into syringe and discharged into a 10-ml tube after the needle was removed. The hemolymph was placed on ice to prevent aggregation of the hemocytes.

PCR

DNA isolation: DNA was isolated using a QIAamp DNA tissue Mini kit (Qiagen, Chatsworth, California), eluted with 30 µl of distilled water, and stored at –20° C until needed.

Primer design and synthesis: Optimal primer sets for amplifying target gene sequences were designed using PC/Gene (Intelligenetics, Mount View, California) and synthesized at the Gene Probe and Expression Laboratory, School of Veterinary

Medicine, Louisiana State University (Table 5-1). The purpose of each primer set was as follows: (1) OYR-1 and OYR-2 to amplify 291 bp of the 28S rRNA gene for identifying PCR inhibitors and the existence of oyster DNA, (2) OYACT-1 and OYACT-2 to amplify 770 bp of actin cDNA or the larger actin gDNA for detecting actin mRNA and the actin intron, (3) GFP-5 and GFP-6 to amplify 539 bp of the gene for red-shifted green fluorescent protein (rsGFP) cDNA for detecting rsGFP mRNA, (4) VEC-1 and VEC-2 to amplify 863 bp of cecropin gDNA sequence for detecting cecropin B gDNA, (5) CECRO-1 and FISH-2 to amplify 1200 bp LOKI-24 cassette for detecting LOKI-24 , and (6) MCEP-1 and MCEP-2 to amplify 135 bp for detecting cecropin B mRNA.

Optimization of PCR: PCR reactions were performed in a 50 μl final volume using a PTC-100^{TM} Programmable Thermal Controller (MJ Research, Watertown, Massachusetts). By optimizing the concentration of magnesium (Perkin Elmer), dimethyl sulfoxide (DMSO)(Sigma Chemical Company, St. Louis, MO), and the annealing temperature, the optimal conditions for each primer set were obtained. For OYR-1, -2, VEC-1, -2, OYACT-1, -2, CECRO-1 and Fish-2 primer sets, the optimal final concentrations of reagents were as follows: 10 μl (~ 0.01 μg) oyster gDNA, 0.5 μM of each primer, 0.01 mM deoxynucleotide triphosphates (dNTPs, Perkin Elmer, Foster City, California), 0.25 mM $MgCl_2$, 1% (v/v) DMSO, 0.025 U AmpliTaq DNA polymerase (Perkin Elmer), 1X AmpliTaq buffer I (Perkin Elmer, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.3). Cycle conditions were as follows: denature at 95° C for 3 min followed by 30 cycles of denaturing at 95° C for 30 sec, annealing at 52° C for 1 min, and extension at 72° C for 1 min. For primer set GFP-5 and GFP-6, the optimal final concentrations of reagents were as follows: 10 μl (~0.01 μg) oyster DNA, 0.1 μM of

Table 5-1. **The nucleotide sequences of primer sets used in PCR and RT-PCR to detect the existence and expression of target genes.**

Primer set (bp)	Target genes	Amplified base pair	Primer sequences
OYR-1 (26)	28S rRNA gDNA	291	5'-GCT-AAA-TAC-TTC-CCG-AGT-CCG-ATA-GC-3'
OYR-2 (24)			5'-GAC-CCT-TCC-TCC-AGC-TCT-TCT-GAC-3'
OYACT-1 (20)	Actin cDNA	770	5'-AGA-ACC-ACC-GAT-CCA-GAC-GG-3'
OYACT-2 (24)			5'-ACA-CTT-TCT-ACA-ATG-AAC-TCC-GTG-3'
GFP-5 (29)	rsGFP cDNA	539	5'-GTC-AGT-GGA-GAG-GGT-GAA-GGT-GAT-GCA-AC-3'
GFP-6 (29)			5'-GAA-AGG-GCA-GAT-TGT-GTG-GAC-AGG-TAA-TG-3'
VEC-1 (24)	Cecropin B gDNA	863	5'-AGA-CTT-GAC-TCC-GCT-GCA-TAA-GTG-3'
VEC-2 (22)			5'-TAC-CGT-TTC-TGA-TGT-TGC-GAC-C -3'
CECRO-1 (29)	LOKI-24 cassette	1200	5'-GCC-GGA-TCC-GTG-TAT-TCC-TGG-ACC-AAA-AA-3'
FISH-2 (25)			5'-TCT-CCT-GAT-GTA-GTG-GCG-TGT-GGT-C-3'
MCEP-1 (26)	Cecropin B mRNA	135	5'-CAG-AAC-CAA-AGC-GAA-CAC-GAA-GAA-AA-3'
MCEP-2 (25)			5'-ATC-GCC-GGT-CAA-GCC-TTG-ACA-ATA-C-3'

each primer, 0.01 mM dNTPs (Perkin Elmer), 0.02 U Vent® DNA polymerase (New England Biolabs, Beverly, Massachusetts), 1X ThermoPol Buffer (New England Biolabs, 10 mM KCl, 20 mM Tris-HCl, pH 8.8 at 25° C, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100). Cycle conditions were as follows: denature at 95° C for 3 min followed by 30 cycles of denaturing at 95° C for 30 sec, annealing at 63° C for 1 min, and extension at 72° C for 1 min.

Analysis of PCR products: PCR products were analyzed using agarose gel electrophoresis. Twenty µl of each PCR product were mixed with 2 µl of tracking dye (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol in distilled water), and electrophoresed in a 1% or 2% Tris-acetate (TAE, 0.04 M Tris-acetate, 1 mM EDTA) agarose gel. The gels were electrophoresed at 5 volts/cm for 120 min, and stained with 0.5 µg ethidium bromide/ml TAE for 15 min, de-stained in TAE for another 15 min and visualized with a UV transilluminator (Pharmacia Inc., Piscataway, New Jersey). A digital camera (DC 120, Kodak, New York) was used to document the image to be analyzed with an Electrophoresis Documentation and Analysis System 120 (Kodak).

Verification of PCR products: PCR products were verified by sequencing. PCR products were separated on TAE agarose gel and stained with ethidium bromide as described above. The gels were visualized with long wave UV light to avoid DNA damage, and a band of the expected size band was excised with a sterile razor blade. An agarose gel DNA extraction column (Qiagen) was used to recover DNA from the agarose. A Cycle Sequencing Ready kit (Perkin Elmer) and an ABI Prism™310 Genetic Analyzer (Perkin Elmer) were used to determine the DNA sequence. Final sequences

were verified by comparison with published sequences using BLAST programs (Altschul et al. 1997, Zhang et al. 1997) that are available at the internet web site of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

RT-PCR

Total RNA isolation and decontamination of DNA: Total RNA was isolated using an RNeasy mini column (Qiagen). Plasmid DNA or gDNA in total RNA was eliminated using Deoxyribonuclease I (Life Technologies, Gaithersburg, Maryland). Twenty-four µl of total RNA were transferred to an RNase-free, 0.3 ml microcentrifuge tube containing 3 µl of 10X DNase I reaction buffer (200 mM Tris-HCl pH 8.4, 20 mM $MgCl_2$, 500 mM KCl), 3 U of DNase I, and incubated at 25° C for 15 min. DNase I was inactivated at 65° C for 10 min in the presence of 2.5 mM EDTA.

Reverse transcription (first strand complement DNA synthesis): First strand cDNA was synthesized using Moloney Murine Leukimia Virus (M-MuLV) reverse transcriptase First–Strand cDNA synthesis Kit (Phamacia) with minor modifications as follow: 8 µl of DNA-free RNA was added to an RNase-free 0.3 ml micro-centrifuge tube, heated 10 min at 65° C and chilled on ice to denature the RNA secondary structure. Five µl of Bulk First-Strand Reaction Mix, 1 µl DTT solution (200 mM), and 1 µl *Not I*-d $(T)_{18}$ primer (0.2 µg) were added. The reaction was incubated at 37° C for 1 h to allow mRNA to be transcribed to single strand DNA (ssDNA).

PCR amplification and verification of RT-PCR products: 15 µl of the ssDNA (above) was used as template for PCR. A primer set for the target gene was used and PCR conditions for each primer set were as described above. Verification and sequencing of RT-PCR products were the same as described above.

Plasmids

The pPC-6 containing cecropin B genomic DNA included 621 bp of cecropin B promoter sequence and 1086 bp of coding and non-coding sequence (Figure 5-1 A). The pQZ-1 containing 621 bp of cecropin B promoter sequence linked with rsGFP reporter gene (Figure 5-1 B)(Zhang et al. 1998), pLOKI-24 contained a synthetic lytic peptide gene, LOKI-24, controlled by the cecropin B promoter (Figure 5-1 C)(Cooper et al. unpublished data).

Plasmid mini-preparations: Plasmids were prepared from 1 ml of culture using mini-preparation methods (Sambrook et al. 1989) and verified by digestion with the proper restriction enzymes and agarose gel electrophoresis. One-ml aliquots of culture were frozen and stored at – 80° C, after the addition glycerol to a final concentration of 20%.

Large scale plasmid preparation: Two ml of culture were expanded to 200 ml using LB medium with the proper antibiotic and incubated for 10 hours at 37° C with shaking. The culture was expanded to 300 ml and incubated for an additional 8 hours. A Qiagen-tip 500 column (Qiagen) was used to isolate plasmid DNA. Purity and concentration were estimated by spectrophotometry (GeneQuant RNA/DNA calculator, model 80-2104-98, Pharmacia Biotech, Cambridge, England). Plasmids were resuspended in Tris EDTA buffer (10 mM Tris, 1 mM EDTA)(TE, pH = 7.4) and stored at –20° C for later use.

Hemocyte Number Determination

Ten µl of hemolymph from each oyster were used for counting hemocyte numbers. Hemocyte number was counted using a hemocytometer at 100 X magnification

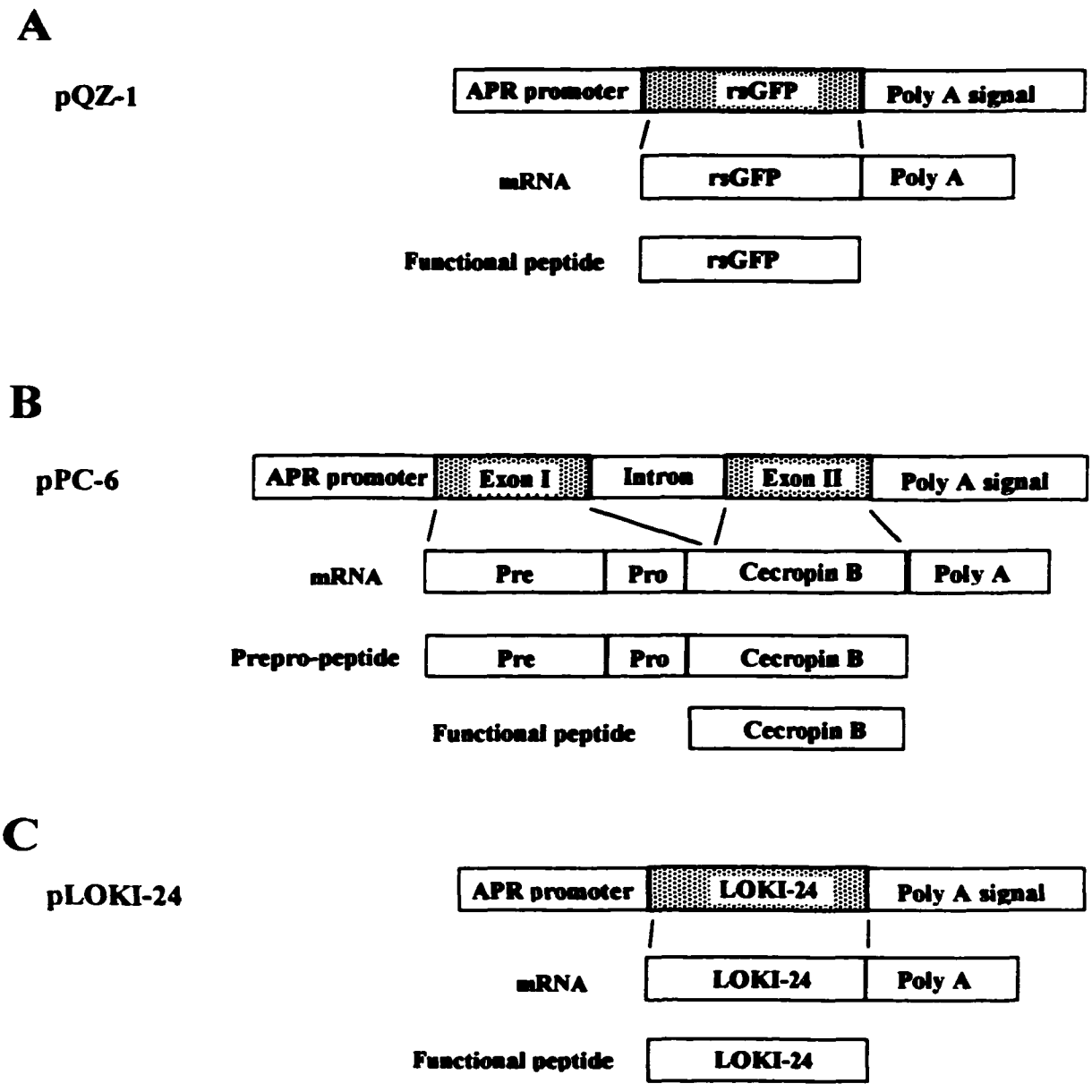

Figure 5-1. The structure of transferred genes and their products. APR: acute phase response cecropin B promoter. rsGFP: red shifted green fluorescent protein. Pre: pre sequence of cecropin B. Pro: pro sequence of cecropin B. LOKI-24: a synthetic antimicrobial peptide gene containing 24 amino acid residues.

with phase contrast microscopy (Optiphot-2, Nikon Inc., Garden City, New York). Hemocyte number per ml hemolymph was calculated.

Hemocyte Killing Ability Assay

The hemocyte killing ability of each oyster was measured using a tetrazolium dye reduction assay (Volety et al. 1999). *Vibrio vulnificus* (1.25×10^6) was used as a target to measure hemocyte killing. Briefly, hemolymph from each oyster was seeded at a density of 1×10^5 cells/well in 6 wells of a 96-well flat-bottom plate so that each oyster could be tested in triplicate; 3 hemocyte wells with bacteria and 3 hemocyte wells without bacteria. Plasma was aspirated after the hemocytes attached to the bottom. After cell attachment, 0.22 µm filtered artificial sea-water (FASW) was added to 3 of the 6 hemocyte wells, 25 µl per well (Hemocytes only, H), and 25 µl of FASW containing *V. vulnificus* (1.25×10^6) were added to the remaining 3 wells (Hemocytes with bacteria, HB). In addition, 3 wells containing 25 µl of FASW (Blank) and 3 wells containing 25 µl *V. vulnificus* (1.25×10^6/ 25 µl FASW) were used as controls. The plates were incubated at 25° C for 3 h for killing to proceed. Thereafter, 100 µl of nutrient broth plus salt (3 g/L beef extract, 5 g/L peptone, and 20 g/L NaCl)(DIFCO Laboratories, Detroit, Michigan) were added to each well and the plate was incubated at 37° C for 2 h to allow the surviving bacteria to grow. To each well was added 20 µl of MTS/PMS reagent (Promega, Madison, Wisconsin) and the plates were incubated at 25° C for 30 min to allow the color to develop. Absorbance (Abs) was measured at 490 nm using an ELISA reader and the Abs of the blank wells was subtracted. The killing index (KI) percentage was calculated as $1 - [(Abs_{HB} - Abs_H)/(Abs_V)] \times 100$.

Bacteria Hemolymph Loading Assay

Drop dilutions were used for the bacteria hemolymph loading assay. One hundred µl of hemolymph from each oyster were serially diluted 10, 100 and 1000 x using 900 µl FASW. Three drops (20 µl/drop) of non-diluted and diluted hemolymph were dropped separately on a marine agar plate (Difco Laboratories). Plates were incubated at 25° C for 16 h for the growth of bacteria colonies. The colony forming units (cfu) per 100µl hemolymph were calculated.

***P. marinus* Tissue Loading Assay**

Each whole oyster meat was weighed and homogenized in a 50-ml tube containing 20 ml of FASW (Fisher and Oliver 1996). One ml of the homogenized tissues was transferred to a 20-ml tube containing 9 ml of Ray's fluid thioglycollate media in triplicate tubes. Then, 50 µl of chloramphenicol (25 mg/ml ethanol)(Sigma) were added and mixed before an addition of 1 ml of nystatin (1 mg/ml distilled water)(Sigma), was layered on the top without mixing. The tubes were incubated in the dark at 25° C for 7 d. Thereafter, the tubes were centrifuged at 1,500 x g for 10 min and the supernatants were discarded. For tissue degradation, 10 ml of 2 M NaOH were added to each tube and incubated at 60° C for 3 h followed by 3 washes with 10 ml of distilled water and centrifugation at 1,500 x g for 10 min. The parasite pellets were resuspended in 1 ml of Lugol's solution (potassium iodide 2.4 mg/ml, iodine 1.6 mg/ml distilled water) and mixed by vortexing for 1 min. One hundred µl of Lugol's solution containing parasites were placed on filter paper (0.22 µm) and aspirated. The parasites stained a dark blue color, were counted under 100 X magnification using a phase-contrast microscope

(Optiphot-2, Nikon Inc., Garden City, New York). The number of parasites per gram tissue was determined.

Bacteria Tissue Loading Assay

Drop dilutions were used to determine bacteria tissue loading. One hundred μl of the supernatant of homogenized tissues from each oyster were diluted to 10, 100 and 1000 X in 900 μl FASW. Three drops (20 μl/drop) of undiluted or diluted supernatants were plated separately on marine agar (Difco Laboratories). The plates were incubated at 25° C for 16 h for the growth of bacteria colonies. The number of colony forming units (cfu) per ml of supernatant was determined.

Study 1: Determining Whether the Gill Actin Gene Contains an Intron and Expresses in Hemocytes

Polymerase chain reaction was performed on hemocyte gDNA using OYACT-1 and -2 primer sets that were designed to amplify a 770 bp fragment of gill actin cDNA. If the actin gene contained an intron between the primers, the size of PCR products would be expected to be greater than 770 base pairs (bp). PCR products were analyzed using a 1% TAE agarose gel. The actual size (bp) and sequence of PCR products were determined as described above. Meanwhile, RT-PCR was performed on total RNA isolated from hemocytes to determine whether the gill actin gene was expressed in hemocytes. The RT-PCR products were subjected to gel electrophoresis followed by sequence determination of the target band excised from a gel. The sequence of the band was compared with gill actin cDNA.

Study 2: Evaluating the Feasibility of the Transfer of Antimicrobial Peptide Genes Controlled by a Cecropin B Promoter to Enhance Oyster Immunity

Before transfection: A total of 60 oysters were randomly divided into 5 groups. Each group contained 12 oysters. Each oyster was assigned a number of one through twelve. Three hundred µl of hemolymph were collected from each oyster for measuring hemocyte number, hemocyte killing, and bacteria hemolymph loading as described above and the mean of each parameter of each group was calculated.

Transfection: Oysters were transfected with pQZ-1, pPC-6, and pLOKI-24 plasmids using SuperFect™ (Qiagen), a commercial transfection reagent. Based on preliminary studies, 10 µg of each plasmid were mixed with SuperFect™ at a ratio of 1:4 (µg/µl) in 150 µl saline (0.48 g/L $CaCl_2$, 1.45 g/L $MgSO_4$, 2.18 g/L $MgCl_2 \cdot 6H_2O$, 0.31 g/L KCl, 11.61 g/L NaCl, 0.35 g/L $NaHCO_3$) and injected into the oyster adductor muscle sinus. Each of the five groups of oysters received a different treatment (injection) (Table 5-2). Transfected oysters were returned to the tanks 6 h after injection.

After transfection: Three days after transfection, hemolymph from each oyster was collected for DNA, RNA, and functional peptide analysis. PCR was performed on DNA isolated from hemocytes using a primer set (OYR-1 and OYR-2, Table 5-1) for the 28S rRNA gene as a positive control. Amplification of 291 bp of 28S rRNA indicated the successful isolation of hemocyte DNA. For the detection of gene transfer into hemocytes, primer sets specific to each transgene (Table 5-1) were used in PCR as follows: GFP-5 and GFP-6 for rsGFP, VEC-1 and VEC-2 for cecropin B, CECRO-1 and FISH-2 for LOKI-24.

RT-PCR was performed on pooled total RNA from each group for the detection of cecropin B mRNA. Oyster actin specific primers, OYACT-1and OYACT-2 (Table 5-1), were used as a positive control. Using primers, MCEP-1 and MCEP-2 (Table 5-1),

Table 5-2. Experimental design for the evaluation of the effect of antimicrobial gene transfer on oyster immunity.

Oyster group (12 oysters/group)	Treatments	Target gene in plasmid*	Purposes	Parameters evaluated for all groups
I	Saline	—	Saline Control	Detection of transferred target genes by PCR
II	SuperFect	—	SuperFect Control	Detection of cecropin B mRNA by RT-PCR
III	pQZ-1	Red shifted green fluorescent protein (rsGFP)	Plasmid Control	The amount change (after transfection – before transfection) of : 1) hemocyte number. 2) hemocyte killing index. 3) bacteria hemolymph loading.
IV	pPC-6	Cecropin B, a genomic antimicrobial peptide gene from moth.	Evaluate cecropin B function	P. marinus tissue loading after transfection.
V	pLOKI-24	LOKI-24, a 24 amino acid synthetic antimicrobial peptide gene	Evaluate LOKI-24 function	Bacteria tissue loading after transfection.

* All target genes were controlled by an acute phase response promoter (from cecropin B gene).
– denotes no plasmid was transferred to the oyster group.

specific to cecropin B mRNA, RT-PCR can amplify a 135 bp band corresponding to cecropin B mRNA. Amplification of the 135 bp band suggests the transcription of cecropin B; RT-PCR was not conducted to detect the LOKI-24 mRNA due to the small gene size (84 bp).

Hemocyte number, hemocyte killing index, and hemocyte bacterial loading were measured. The amount change of each parameter was calculated by subtracting the value of each parameter measured before transfection from the value of each parameter measured after transfection. Thereafter, oysters were used for *P. marinus* tissue loading and bacteria tissue loading assays described above and the mean of each parameter of each group was calculated.

Data Analysis

For detecting significant differences among groups of evaluated parameters, the hemocyte number, hemocyte killing index, bacteria hemolymph loading, *P. marinus* tissue loading and bacterial tissue loading were analyzed. Due to the non-normal distribution of the data after transformation, the non-parametric Kruskal-Wallis test was used to detect the significant different ($P < 0.05$).

Results

Study 1: Determining Whether the Gill Actin Gene Contains an Intron and Expresses in Hemocytes

Based on agarose gel electrophoresis, PCR products from hemocyte gDNA and RT-PCR from hemocyte total RNA were about 770 bp, similar to gill actin cDNA (Figure 5-2). To distinguish gDNA from actin cDNA using agarose gel electrophoresis was not possible. By DNA sequencing, the PCR product of hemocyte DNA showed 726

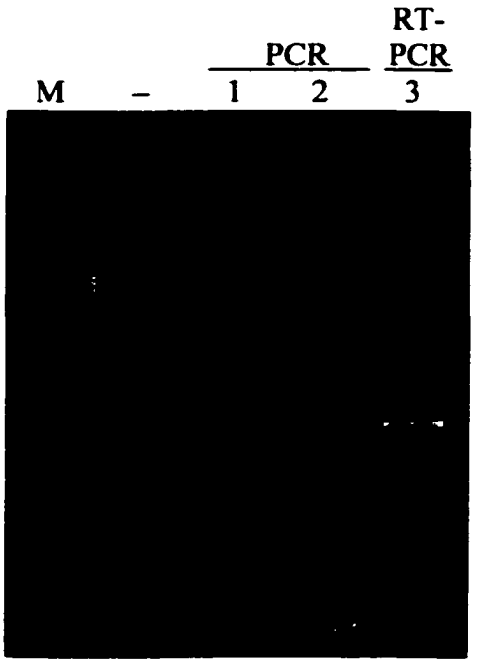

Figure 5-2. Agarose gel analysis of PCR and RT-PCR products from hemocytes. Using OYACT-1 and - 2 primer sets, PCR was performed on 10 ng genomic DNA (lane 1) and total RNA digested with DNase I (lane 2), and RT-PCR was performed on the total RNA digested with DNase I (lane 3). A 770 bp band in lane 1 indicates the actin gene was amplified. No band was found in lane 2 indicating no genomic DNA contamination. A band (~770 bp) in lane 3 indicates the actin mRNA was amplified. M: 100 bp marker, –: PCR negative control without DNA.

bp (not including 44 bp of primer sequence), and 93% identity (agreement) with the published gill actin gene cDNA (Table 5-3A).

Gel analysis of RT-PCR products of total RNA showed similar size to PCR products of gDNA (Figure 5-2). The nucleotide sequence of RT-PCR products of hemocyte RNA showed 726 bp and 100% homology when compared with the published actin cDNA sequences (Table 5-3B), indicating that the actin gene was expressed in hemocytes.

Study 2: Evaluating of the Feasibility of the Transfer of Antimicrobial Peptide Genes Controlled by a Cecropin B Promoter to Enhance Oyster Immunity

The 28S rRNA gene was amplified from all DNA isolated from oysters of each group (Figure 5-3 A to E). The results indicated successful DNA extraction and that PCR performed as expected. Genes corresponding to rsGFP, cecropin B, and LOKI-24 were not detected in oyster groups receiving saline (Figure 5-4 A; 5-5 A and 5-6 A) or SuperFect™ only (Figure 5-4 B; 5-5 B and 5-6 B) but were detected in oysters transfected with pQZ-1 (Figure 5-4 C), pPC-6 (Figure 5-5 C), and pLOKI-24 (Figure 5-6 C). In group III oysters transfected with pQZ-1 and group V oysters transfected with pLOKI-24, rsGFP and LOKI-24 gene were detected in all 12 oysters of each group (Figure 5-4 C and 5-6 C). Since one oyster (the eleventh) died after transfection in group IV, data from the eleventh oyster in each of the other groups was excluded from data analysis. The cecropin B gene was detected in all 11 oysters transfected with pPC-6 (Figure 5-5 C)(Table 5-4). The PCR positive oysters indicated the plasmids were retained in oysters hemolymph.

The mRNA of cecropin B was not detected in the hemocytes of pPC-6 transfected oysters. No contamination of actin genomic DNA (Figure 5-7 A) and cecropin B plasmid

Table 5-3 A. **Nucleotide sequence comparison of PCR product and gill actin cDNA. The identity (agreement) was 682/726 (93%).**

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PCR:    1   tagccccagaggagcaccccgtcctcctgaccgaggcccatctcaaccccaaggccaaca 60
            |||||||||||||||||||||||||| |||||||||||| ||||||||||||||||||||
cDNA:   40  tagccccagaggagcaccccgtcctcttgaccgaggcccctctcaaccccaaggccaaca 99

PCR:    61  gagaaaagatgacacagatcatgttcgaaaccttcaactctcccgccatgtacgtcgcca 120
            |||||||||||||||||||||||||| |||||||||| ||||||||||||||||||||
cDNA:   100 gagaaaagatgacacagatcatgttcgagaccttcaactcccccgccatgtacgtcgcca 159

PCR:    121 tccaggccgtactgtccctgtacgcttccggtcgtaccactggtatcgtactcgactccg 180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
cDNA:   160 tccaggccgtactgtccctgtacgcttccggtcgtaccactggtatcgtactcgactccg 219

PCR:    181 gagatggtgtgtcccacacagtccccatctacgagggatacgcccttccccacgccatca 240
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PCR:    241 tgagattggatctcgctggacgtgatctgaccgattacctcatgaagatccttacagagc 300
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cDNA:   280 tgagattggatctcgctggacgtgatctgaccgattacctcatgaagatccttacagagc 339

PCR:    301 gtggttactctttcaccaccacagctgagagagaaatcgtcagagacatcaaggagaaac 360
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cDNA:   340 gtggttactctttcaccaccacagctgagagagaaatcgtcagagatatcaaggagaagc 399

PCR:    361 tgtgctatgttgctttggactttgagcaggagatggccactgccgcatcatcatcatccc 420
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PCR:    421 tagagaagagctacgaacttcccgacggtcaggtcatcaccattggcaacgagcgattca 480
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PCR:    721 aatact 726
            ||||||
cDNA:   760 aatact 765
```

Table 5-3 B. **Nucleotide sequence comparison of RT-PCR product and gill actin cDNA. The identity (agreement) was 726/726 (100%).**

```
RT-PCR: 1   tagccccagaggagcaccccgtcctcttgaccgaggcccctctcaaccccaaggccaaca 60
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cDNA:   40  tagccccagaggagcaccccgtcctcttgaccgaggcccctctcaaccccaaggccaaca 99

RT-PCR: 61  gagaaaagatgacacagatcatgttcgagaccttcaactcccccgccatgtacgtcgcca 120
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cDNA:   100 gagaaaagatgacacagatcatgttcgagaccttcaactcccccgccatgtacgtcgcca 159

RT-PCR: 121 tccaggccgtactgtccctgtacgcttccggtcgtaccactggtatcgtactcgactccg 180
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cDNA:   160 tccaggccgtactgtccctgtacgcttccggtcgtaccactggtatcgtactcgactccg 219

RT-PCR: 181 gagatggtgtgtcccacacagtccccatctacgagggatatgcccttccccacgccatca 240
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cDNA:   220 gagatggtgtgtcccacacagtccccatctacgagggatatgcccttccccacgccatca 279

RT-PCR: 241 tgagattggatctcgctggacgtgatctgaccgattacctcatgaagatccttacagagc 300
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
cDNA:   280 tgagattggatctcgctggacgtgatctgaccgattacctcatgaagatccttacagagc 339

RT-PCR: 301 gtggttactctttcaccaccacagctgagagagaaatcgtcagagatatcaaggagaagc 360
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
cDNA:   340 gtggttactctttcaccaccacagctgagagagaaatcgtcagagatatcaaggagaagc 399

RT-PCR: 361 tgtgctatgttgctttggacttcgagcaggagatggccacagccgcttcctcatcctccc 420
        ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
cDNA:   400 tgtgctatgttgctttggacttcgagcaggagatggccacagccgcttcctcatcctccc 459

RT-PCR: 421 tggagaagagctacgaacttcccgacggtcaggtcatcaccattggcaacgaacgattca 480
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cDNA:   460 tggagaagagctacgaacttcccgacggtcaggtcatcaccattggcaacgaacgattca 519

RT-PCR: 481 ggtgcccagaggccatgttccagccatccttccttggtatggaatccgccggtatccatg 540
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cDNA:   520 ggtgcccagaggccatgttccagccatccttccttggtatggaatccgccggtatccatg 579

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RT-PCR: 601 atattgtcctctctggaggtaccaccatgttcccaggtatcgccgacagaatgcagaagg 660
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RT-PCR: 661 aagtcaccaccctcgctcctccaacaatgaagatcaaggtcattgccccacccgagagga 720
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cDNA:   700 aagtcaccaccctcgctcctccaacaatgaagatcaaggtcattgccccacccgagagga 759

RT-PCR: 721 aatact 726
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cDNA:   760 aatact 765
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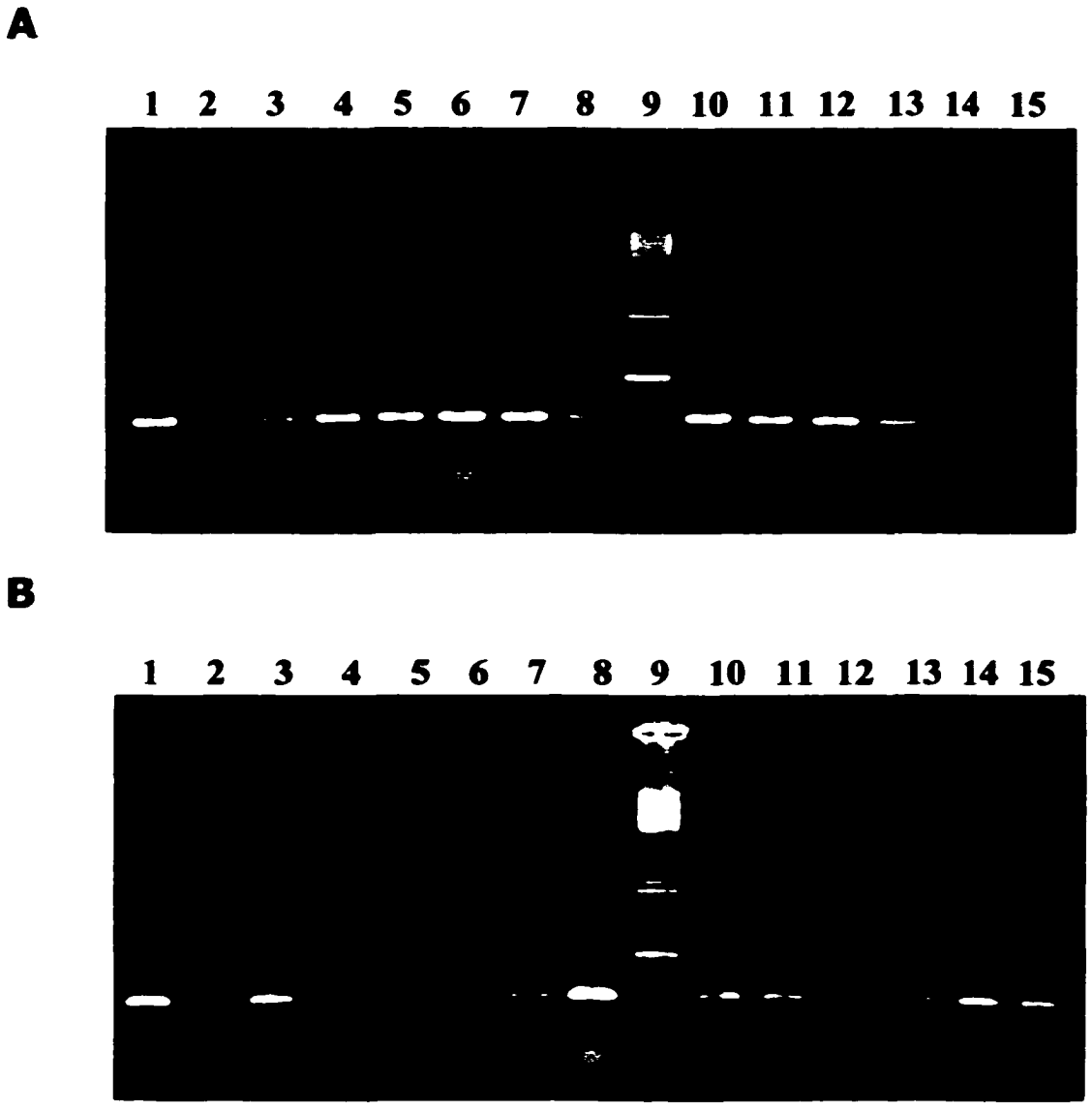
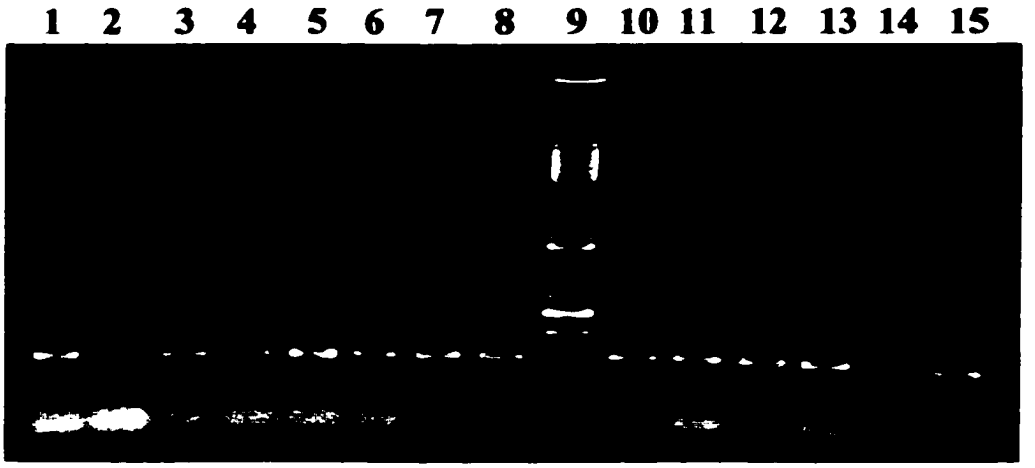


Figure 5-3. Gel electrophoresis of PCR-amplified products using a primer set for 28S rRNA. The amplification of 291bp of 28S RNA as a positive control for PCR and the presence of oyster DNA. Panel A: group I oysters transfected with saline. Panel B: group II oysters transfected with Superfect™. Lane 1: positive control; lane 2: negative control without oyster DNA; lane 3-8 and 10-15: 12 oysters in each group; lane 9: 100-bp marker.

C



D

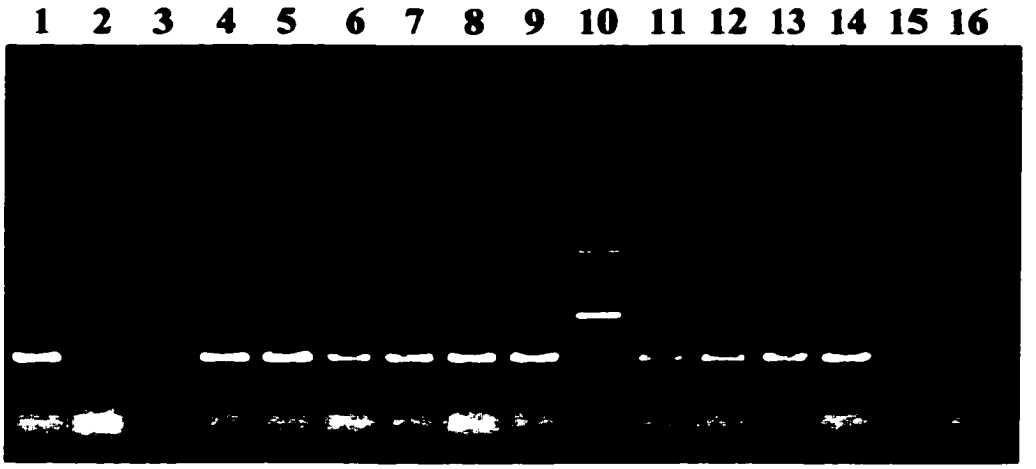


Figure 5-3. Continued. Panel C: group III oysters transfected with pQZ-1. Lane 1: positive control; lane 2: negative control without oyster DNA; lane 3-8 and 10-15: 12 oysters in each group; lane 9: 100-bp marker. Panel D: group IV oysters transfected with pPC-6. Lane 1: positive control; lane 2: negative control without oyster DNA; lane 3: no sample loading to avoid cross contamination to negative control lane 2; lane 4-9, 11-14 and 16: 11 oysters in the group; lane 15: empty lane due the death of the eleventh oyster after transfection; lane 10: 100-bp marker.

E

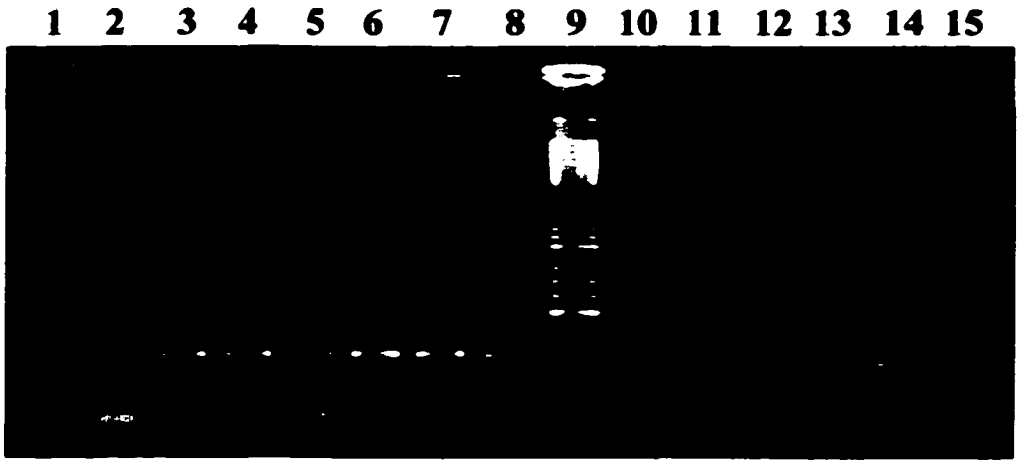


Figure 5-3. Continued. Panel E: group V oysters transfected with pLOKI-24. Lane 1: positive control; lane 2: negative control without oyster DNA; lane 3-8 and 10-15: 12 oysters in each group; lane 9: 100-bp marker.

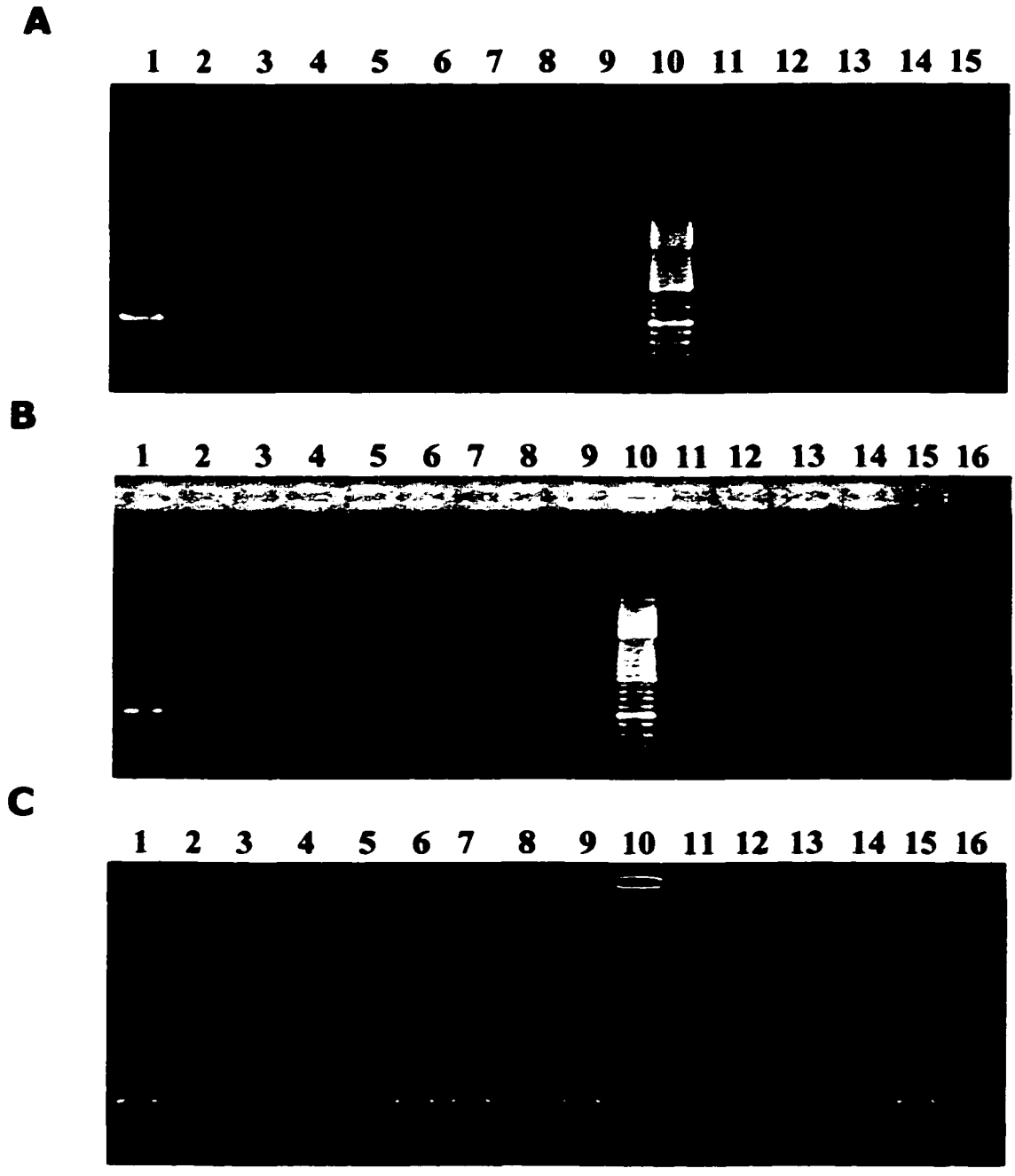


Figure 5-4. Gel electrophoresis of PCR-amplified products using a primer set for rsGFP to identify the existence of the rsGFP gene. The amplification of a 539 bp band of rsGFP gene indicates the presence of rsGFP gene. Panel A: group I oysters transfected with saline. Lane 1: positive control; lane 2: negative control without oyster DNA; lane 3-9 and 11-15: 12 oysters in each group; lane 10: 100-bp marker. Panel B: group II oysters transfected with Superfect™. Panel C: group III oysters transfected with pQZ-1. Lane 1: positive control; lane 2: negative control without oyster DNA; lane 3: empty lane to avoid cross contamination of lane 2 to negative control; lane 4-9 and 11-16: 12 oysters in each group; lane 10: 100-bp marker.

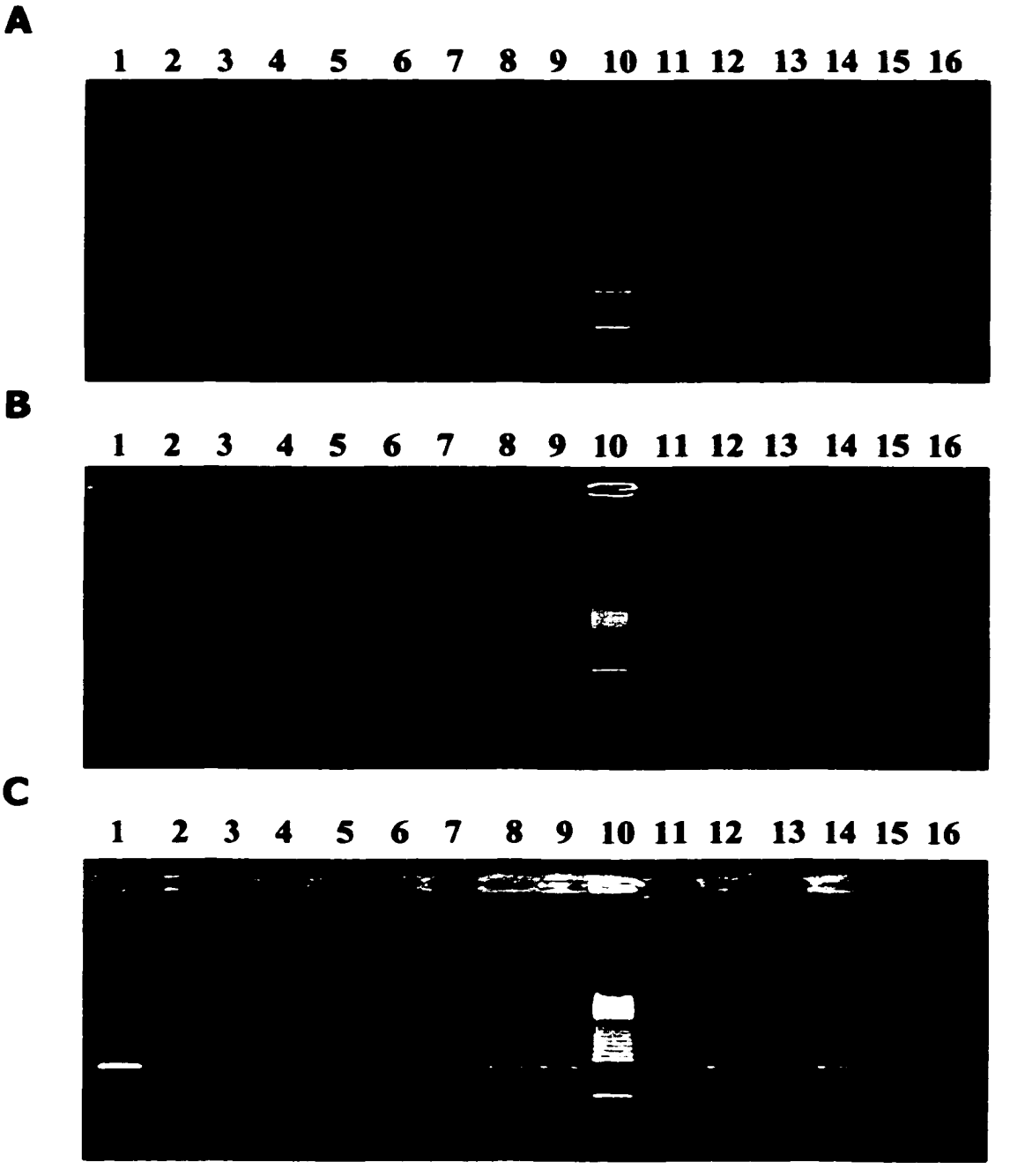


Figure 5-5. Gel electrophoresis of PCR-amplified products using a primer set for the cecropin B gene to amplify an 863 bp band of cecropin B indicating the presence of cecropine B gene. Panel A: group I oysters transfected with saline. Panel B: group II oysters transfected with Superfect™. Panel C: group IV oysters transfected with pPC-6. Lane 1: positive control; lane 2: negative control without oyster DNA; lane 3: empty lane to avoid cross contamination of lane 2 to negative control; lane 4-9, 11-16 : 12 oysters in the group; lane 10: 100-bp marker. Due to the death of the eleventh oyster after pPC-6 transfection, lane 15 of pane C containing no sample.

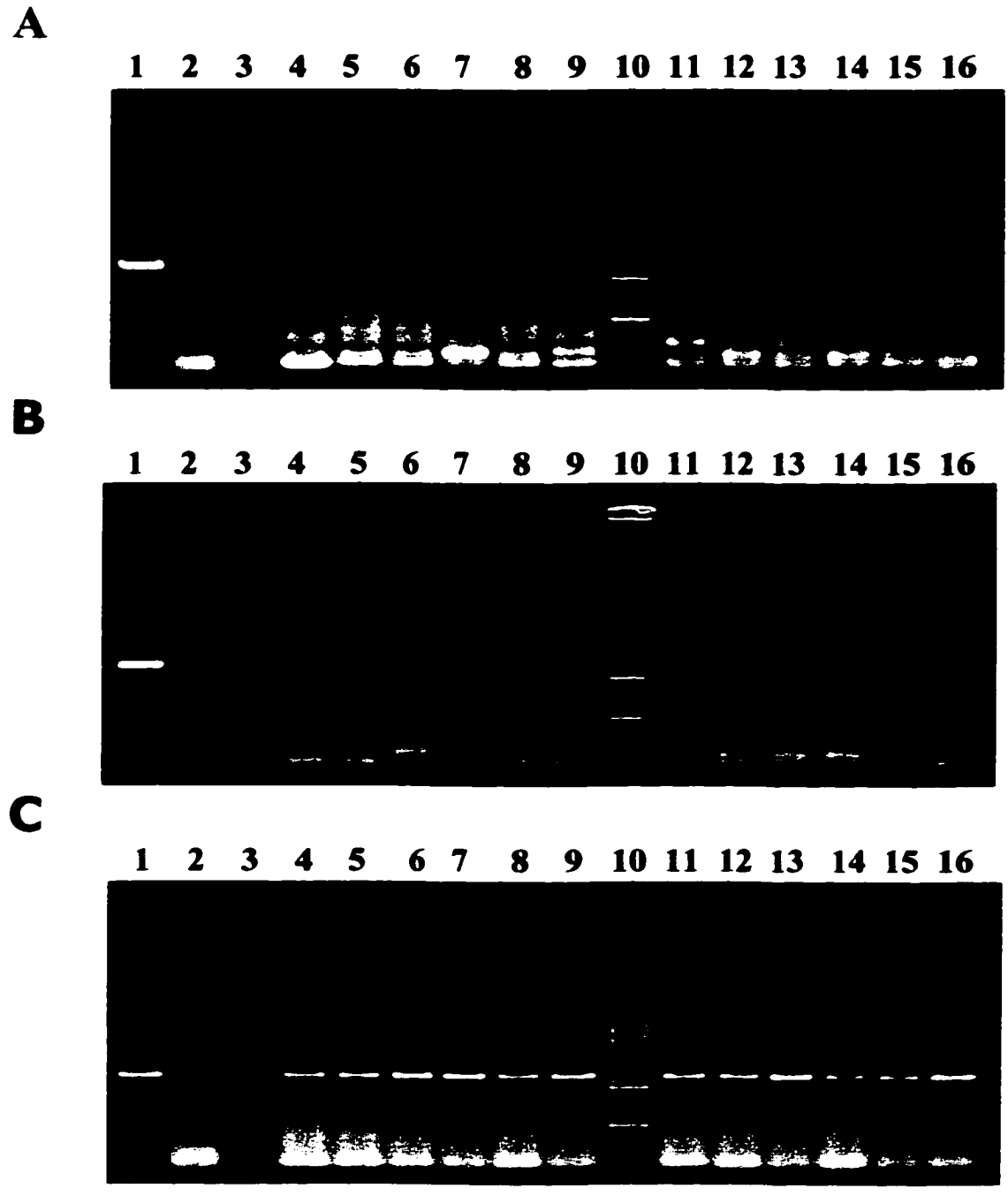


Figure 5-6. Gel electrophoresis of PCR-amplified products using primers for LOKI-24 to amplify 1200 bp of the LOKI-24 gene cassette. Panel A: group I oysters transfected with saline. Panel B: group II oysters transfected with Superfect™. Panel C: group V oysters transfected with pLOKI-24. Lane 1: positive control; lane 2: negative control without oyster DNA; lane 3: empty lane to avoid cross contamination to negative control lane 2; lane 4-9, 11-16 : 12 oysters in the group; lane 10: 100-bp marker.

Table 5-4. Summary of PCR, RT-PCR, and evaluated parameters.

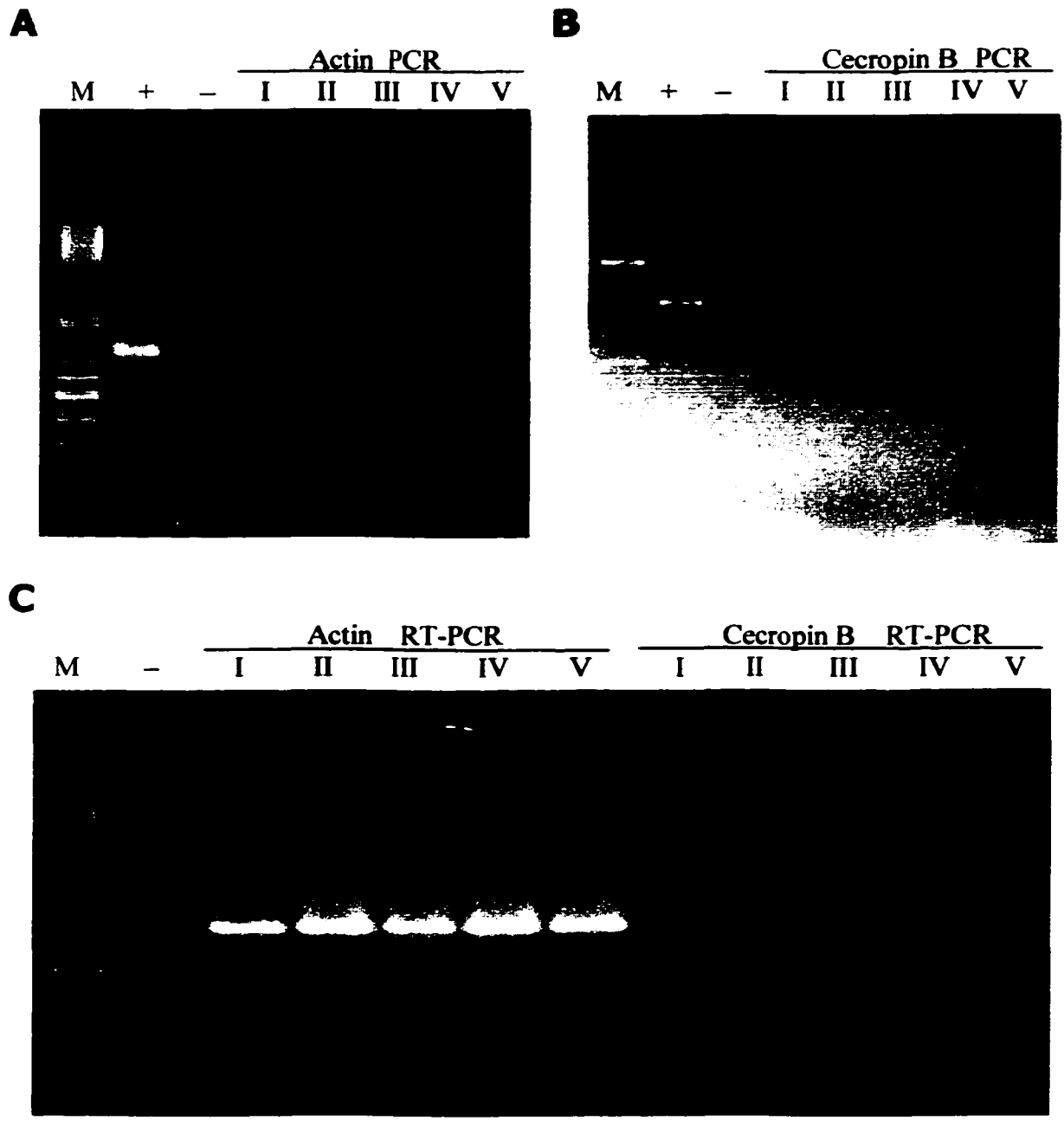
Treatment	PCR positive				RT-PCR positive		Hemocyte number (10^6/ ml)*	Hemocyte killing index (%)*	Bacteria hemolymph loading (10^3cfu/ 100 µl)*	P. marinus tissue loading (10^6/g tissue) **	Bacteria tissue loading (10^3 cfu/g tissue)**
	28S rRNA	rsGFP	Cep B	LOKI -24	Actin	CepB					
Saline	12/12	0/12	0/12	0/12	Yes	No	1.5 ± 2.9	0.9 ± 33	- 0.7 ± 1.1	2.2± 3.3	16.6 ± 8.2
SuperFect™	12/12	0/12	0/12	0/12	Yes	No	0.7 ± 2.0	- 49 ± 46	- 3.5± 8.1	1.3± 1.5	14.8 ± 6.9
pQZ-1	12/12	12/12	—	—	Yes	No	0.8 ± 3.0	- 53 ± 31	22.5 ± 70.1	3.8 ± 6.6	14.1 ± 11.1
pPC-6	11/11	—	11/11	—	Yes	No	0.8 ± 2.6	- 54 ± 32	31.8 ± 107.8	3.0 ± 7.2	11.1 ± 6.7
pLOKI-24	12/12	—	—	12/12	Yes	No	0.3 ± 1.2	- 40 ± 41	0.2 ± 1.1	3.1 ± 6.3	12.8 ± 5.9

* The values indicated the amount change after transfection by subtracting the value of parameter before transfection from the value of parameter after transfection.

** The values indicated after transfection.

— indicated PCR was not performed.

Figure 5-7. Gel analysis of PCR and RT-PCR products from hemocyte total RNA. The total RNA from each oyster within each group was pooled, digested with DNase and subjected to PCR with primers specific to actin (Panel A) and cecropin B (Panel B). RT-PCR was performed on the total RNA of 5 groups of oysters with primers specific to actin and cecropin B mRNA (Panel C). Amplification of a 770 bp band from actin PCR or a 863 bp from cecropin B PCR indicates the contamination of genomic DNA or plasmid DNA of pPC-6. Amplification of a 770 bp band from actin RT-PCR indicates the successful of RT-PCR. Amplification of a 135 bp from cecropin B RT-PCR indicates the transcription of cecropin B gene. M: 100 bp marker. +: positive control of PCR using 0.1μg oyster DNA (Panel A) or 1 ng pPC-6 (Panel B). −: negative control without DNA (Panel A and B) or total RNA (Panel C). I: group I oysters transfected with saline. II: group II oysters transfected with SuperFect. III: group III oysters transfected with pQZ-1. IV: group IV oysters transfected with pPC-6. V: group V oysters transfected with pLOKI-24.



DNA (Figure 5-7B) was detected in total RNA after DNase I digestion. RT-PCR successfully amplified the actin mRNA from total RNA in all five groups indicating the success of RT-PCR but the mRNA of cecropin B was not found (Figure 5-7 C)(Table 5-4).

For the hemocyte number assay, no significant difference ($P < 0.05$) was found in hemocyte numbers among the five groups before and after transfection. Before transfection, the hemocyte numbers of individual oysters showed high variability in group IV (receiving pPC-6)($2.0 \pm 1.7 \times 10^6$ cell/ml, range = 6.0×10^6), and in group V (receiving pLOKI-24)($1.9 \pm 1.2 \times 10^6$ cell/ml, range = 4.1×10^6). After transfection, the hemocyte number of each oyster group increased. The hemocyte number in the saline transfected group showed the largest increase of $1.5 \pm 2.8 \times 10^6$ (range = 10.5×10^6) from $1.5 \pm 0.8 \times 10^6$ cell/ml to $3.0 \pm 2.7 \times 10^6$ cell/ml but no significant difference was found among five groups (Figure 5-8)(Table 5-4 and 5-5). The percent change in hemocyte killing among the five groups showed no significant difference. The hemocyte killing index of each group before transfection was about the same ($68 \pm 34\%$, range = 84% for SuperFect™ transfected group, $70 \pm 33\%$, range = 77% for pQZ-1 transfected group, $68 \pm 28\%$, range = 73% for pPC-6 transfected group, $66 \pm 24\%$, range = 75% for pLOKI-24 transfected group), except for the saline group which showed low activity ($39 \pm 28\%$, range = 90%)(Figure 5-9). After transfection, the hemocyte killing index of the saline transfected group remained constant ($40 \pm 30\%$, range = 90%) while those of other groups showed a 40% to 55% decrease (Figure 5-9)(Table 5-4 and 5-5).

For the bacteria hemolymph loading assay, no significant differences ($P < 0.05$) in the percentage change of bacteria hemolymph loading were found among oyster groups.

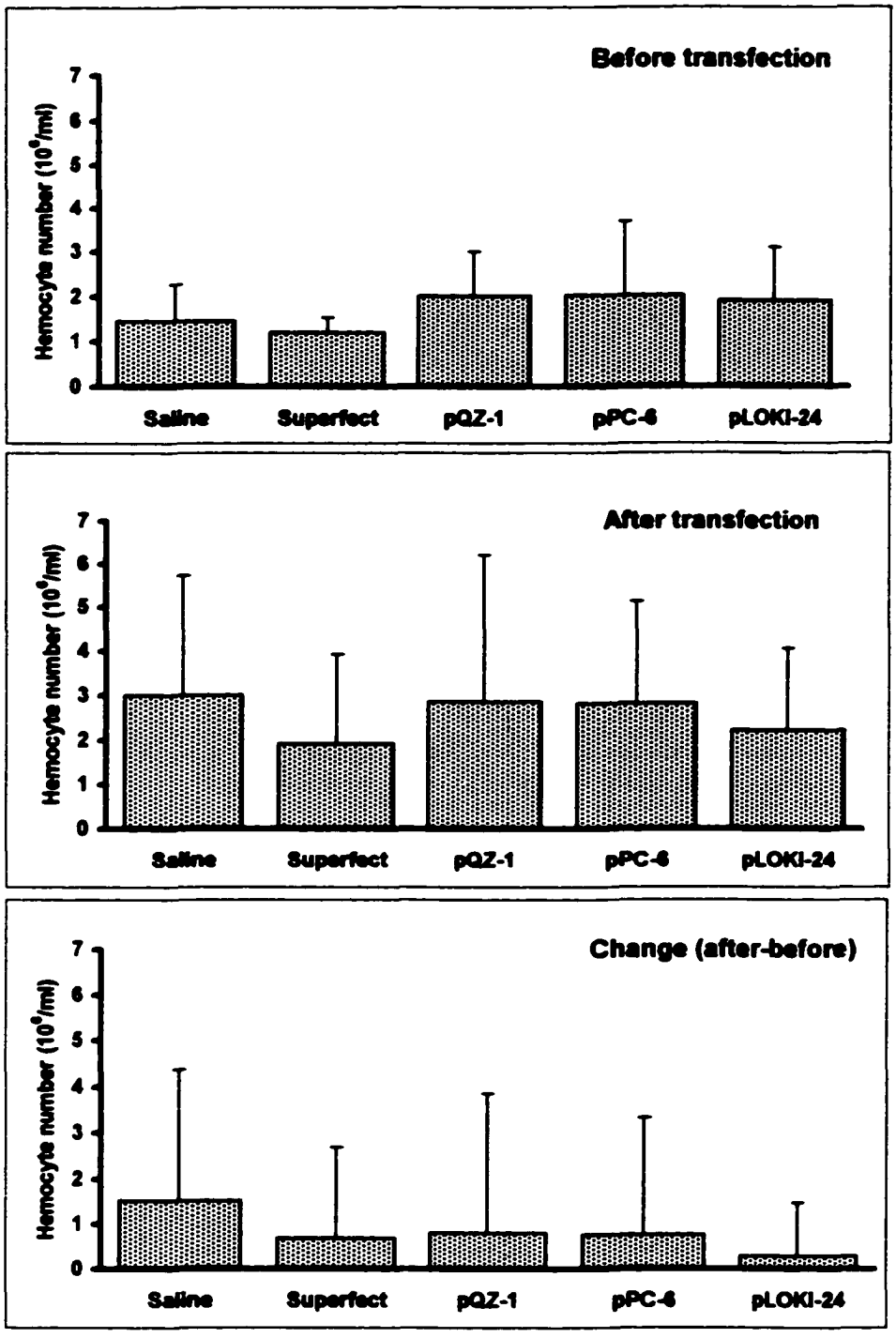


Figure 5-8. Comparison of the effect of antimicrobial peptide gene transfer on oyster hemocyte number (n = 11).

Table 5-5. Summary of evaluated parameters of each treatment (n = 11).

Parameters/Treatments	Mean ± Standard Deviation	Range	Minimum	Maximum	Coefficient of Variation (%)
Hemocyte number (10^6 cell/ml)*					
Saline	1.5 ± 2.8	10.5	- 0.9	9.7	187
SuperFect™	0.7 ± 2.0	7.2	- 0.7	6.5	286
pQZ-1	0.8 ± 3.0	11.1	- 1.9	9.3	375
pPC-6	0.8 ± 2.6	10.8	- 3.8	7.0	325
pLOKI-24	0.3 ± 1.2	3.6	- 1.4	2.2	400
Hemocyte killing index (%)*					
Saline	0.9 ± 32.7	125.5	- 74.7	50.8	3633
SuperFect™	- 48.9 ± 46.4	134.7	-92.7	42.0	-95
pQZ-1	- 52.6 ± 30.8	78.2	- 85.4	- 7.2	-59
pPC-6	- 54.5 ± 32.1	86.5	- 83.2	3.3	-59
pLOKI-24	- 40.2 ± 41.1	147.4	- 85.4	62.0	-102
Bacteria hemolymph loading (10^5 cfu/100 µl)*					
Saline	- 0.7 ± 1.1	3.0	- 2.8	0.2	-157
SuperFect™	- 3.5 ± 8.1	23.8	-21.7	2.1	-231
pQZ-1	22.5 ± 70.1	236.3	- 3.0	233.3	312
pPC-6	31.8 ± 107.8	368.9	-12.3	356.6	339
pLOKI-24	0.2 ± 1.1	4.4	- 1.7	2.8	550
P. Marinus tissue loading (10^6/g tissue)**					
Saline	2.2 ± 3.3	10.4	0.002	10.4	150
SuperFect™	1.3 ± 1.5	4.7	0.0009	4.7	115
pQZ-1	3.8 ± 6.6	22.7	0.008	22.7	174
pPC-6	3.0 ± 7.2	24.2	0.0004	24.2	240
pLOKI-24	3.1 ± 6.3	21.4	0.0002	21.4	203
Bacteria tissue loading (10^3 cfu/g tissue)**					
Saline	16.6 ± 8.2	29.4	4.2	33.6	49
SuperFect™	14.8 ± 6.9	24.1	4.2	28.4	47
pQZ-1	14.1 ± 11.1	36.6	2.3	39.0	79
pPC-6	11.7 ± 6.7	20.3	2.0	22.3	57
pLOKI-24	12.8 ± 5.9	18.4	3.2	21.6	46

* indicates the amount change by subtracting the value before transfection from the value after transfection).

** indicates after transfection.

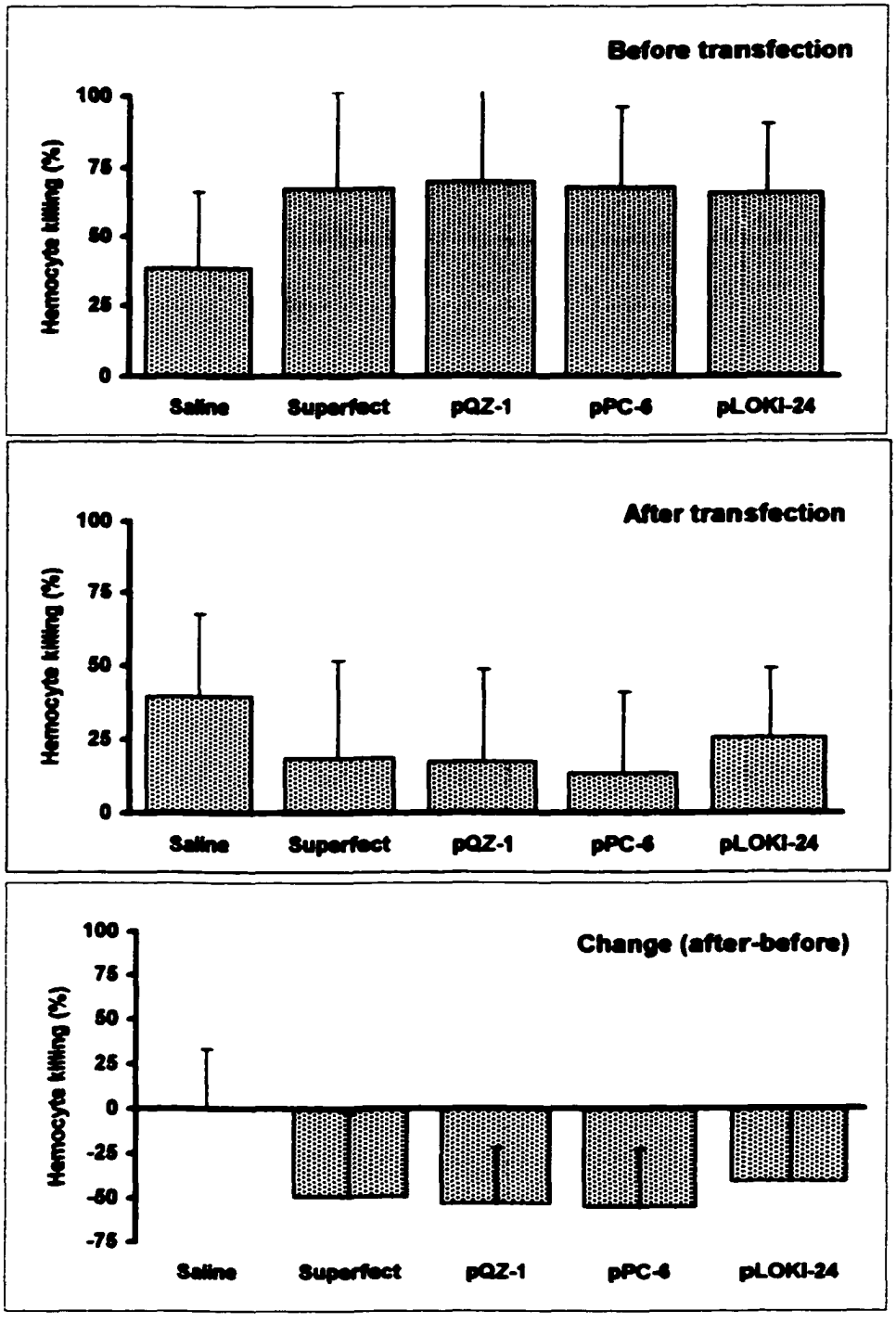


Figure 5-9. Comparison of the effect of antimicrobial peptide gene transfer on oyster hemocyte killing index (n = 11).

Highly variable bacteria hemolymph loading was found among all oysters within each group. The most variable bacteria hemolymph loading was found in pPC-6 group before transfection ($1.3 \pm 3.5 \times 10^8$ cfu/100 µl hemolymph, range = 12.3×10^8). The change of bacteria hemolymph loading increased in the pQZ-1 transfected group ($22.5 \pm 70.1 \times 10^8$, range = 236.3×10^8), pPC-6 transfected group ($31.8 \pm 107.8 \times 10^8$ cfu/100 µl hemolymph, range = 368.9×10^8) and pLOKI-24 (Figure 5-10)(Table 5-4 and 5-5).

For the *P. marinus* tissue loading assay, no significant decrease ($P < 0.05$) of *P. marinus* tissue loading was found among groups. The pQZ-1 transfected oyster groups showed the highest *P. marinus* loading ($3.8 \pm 6.6 \times 10^6$ /g tissue, range = 22.7×10^6), while the SuperFect™ injected group showed the lowest *P. marinus* loading ($1.3 \pm 1.5 \times 10^6$ cfu/g tissue, range = 4.8×10^6) than other groups (Figure 5-11 A)(Table 5-4 and 5-5).

For the bacteria tissue loading assay, no significant decrease ($P < 0.05$) of bacteria tissue loading was found among groups but the bacteria tissue loading decreased in pPC-6 ($11.1 \pm 6.7 \times 10^3$ cfu/g tissue, range = 20.3×10^3) and pLOKI-24 ($12.8 \pm 5.9 \times 10^3$ cfu/g tissue, range = 18.4×10^3) transfected groups compared to the saline injection group ($16.6 \pm 8.2 \times 10^3$ cfu/g tissue, range = 29.4×10^3), SuperFect™ transfected group ($14.8 \pm 6.9 \times 10^3$ cfu/g tissue, range = 24.1×10^3), and pQZ-1 transfected group ($14.1 \pm 11.1 \times 10^3$ cfu/g tissue, range = 36.6×10^3)(Figure 5-11 B and 5-12)(Table 5-4 and 5-5).

Discussion

Analysis of actin genes to determine intron location and tissue expression patterns is important if using actin as an internal control for mRNA quantification, reverse transcription and cDNA library construction. Meanwhile *in vivo* antimicrobial peptide gene transfer might facilitate the development of disease resistance oysters.

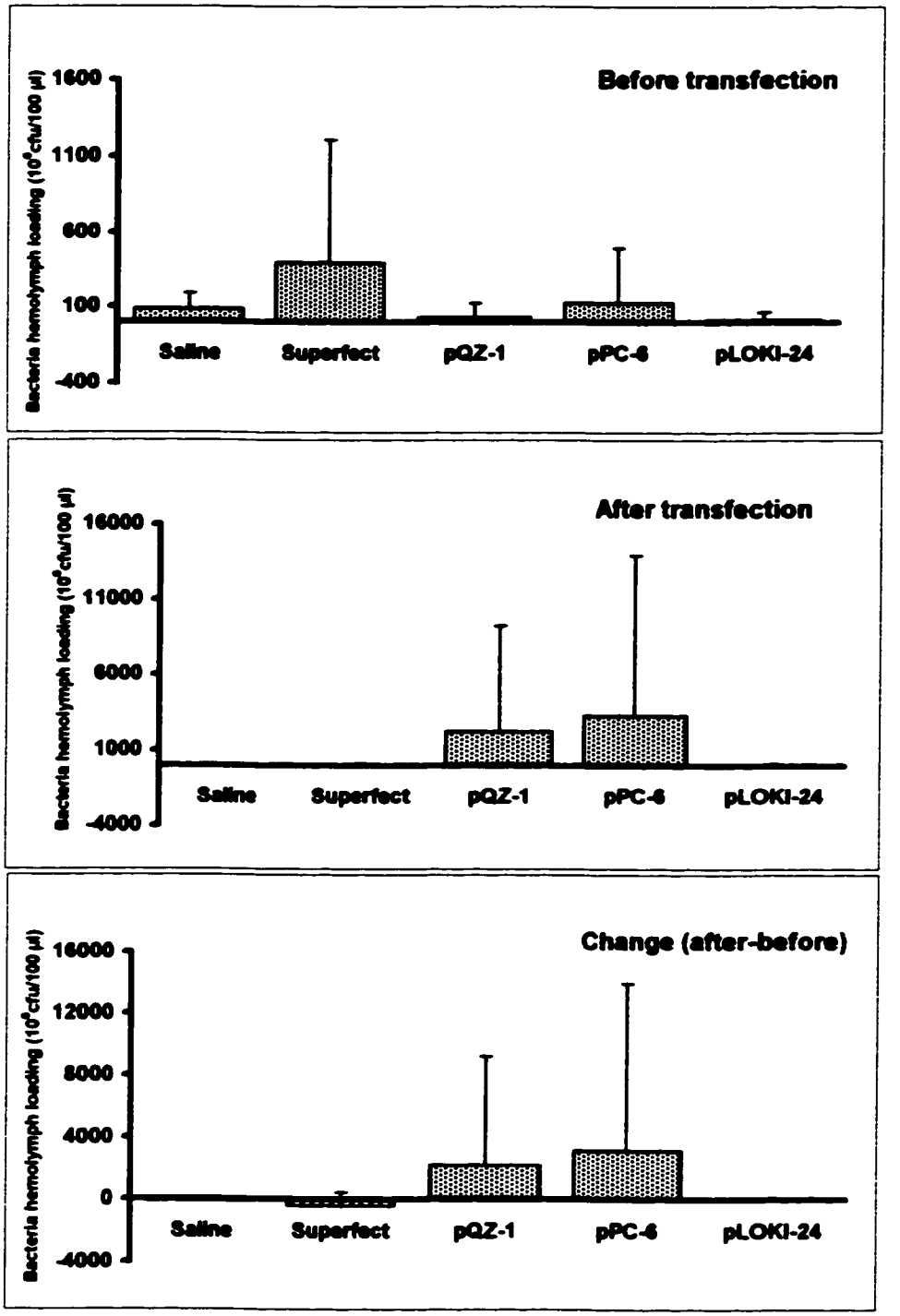


Figure 5-10. Comparison of the effect of antimicrobial peptide gene transfer on oyster bacteria hemolymph loading (n = 11).

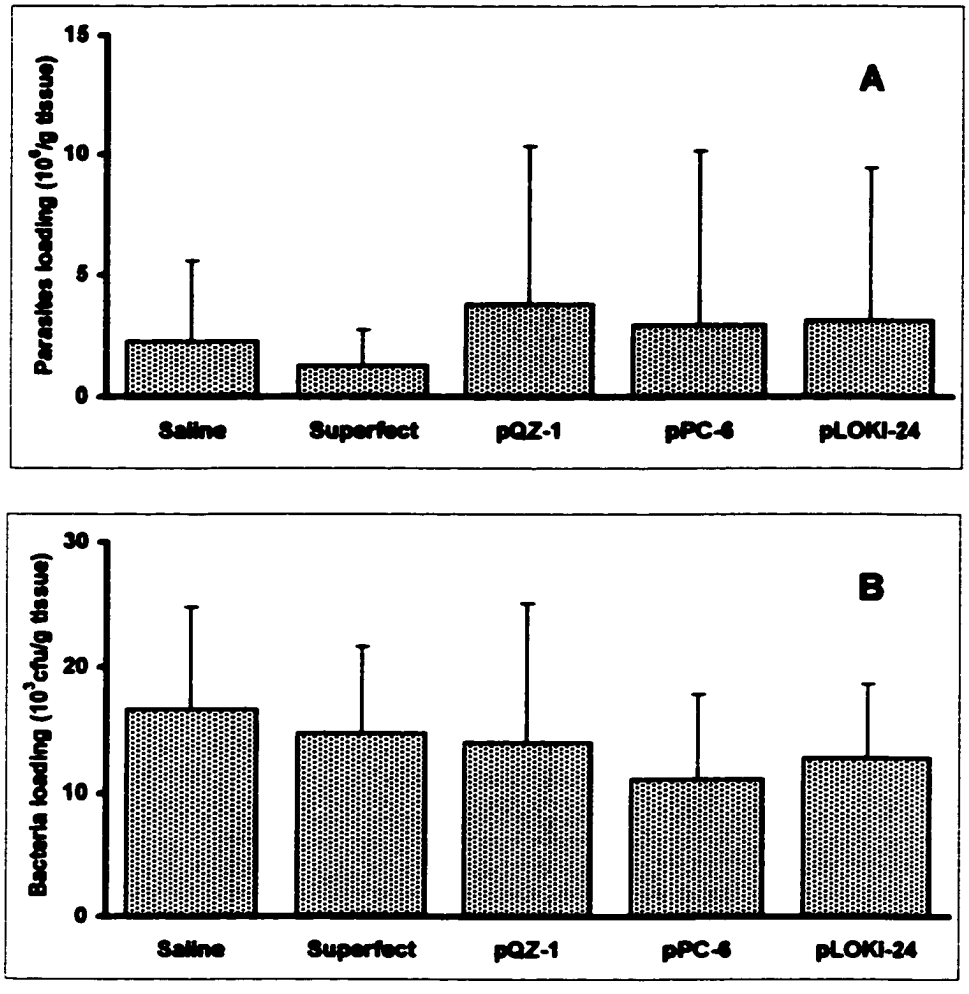


Figure 5-11. Comparison of the effect of antimicrobial peptide gene transfer on *Perkinsus marinus* tissue loading (A) and bacteria tissue loading (B) (n = 11).

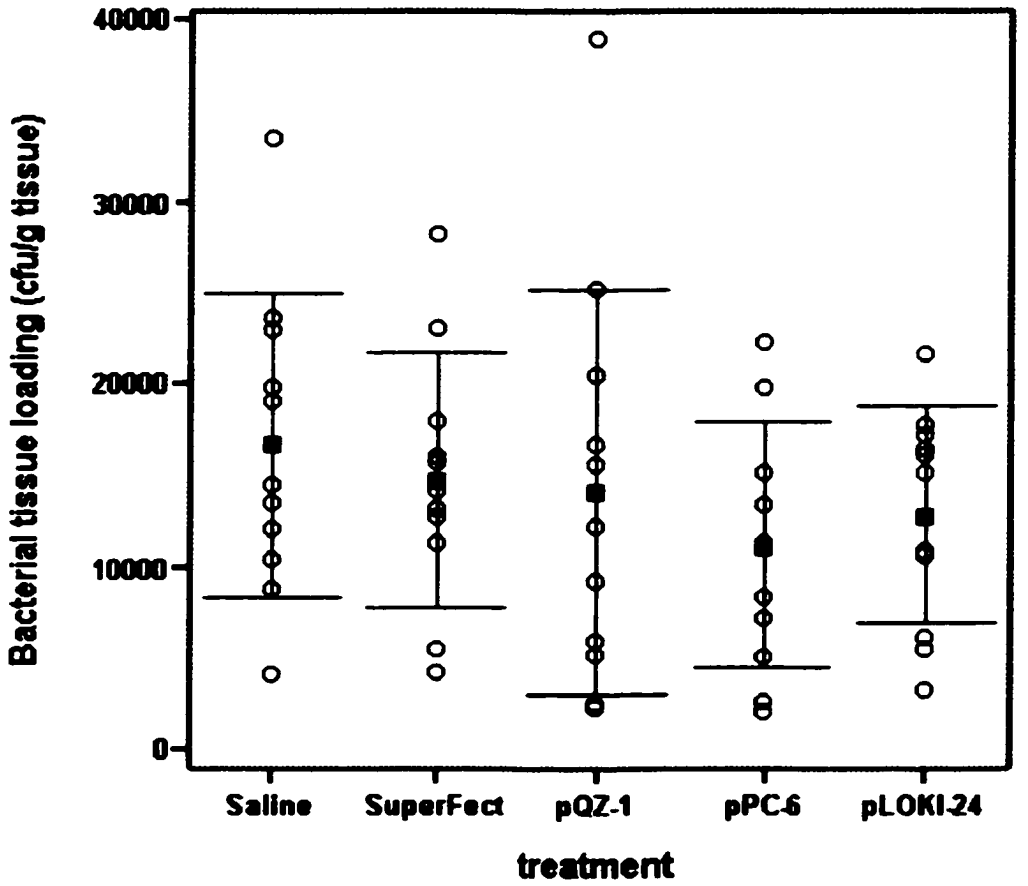


Figure 5-12. Comparison of the effect of antimicrobial peptide gene transfer on bacteria tissue loading. Square with bar indicates mean and standard deviation (n = 11) of each oyster group. Circle indicates mean of individual oysters from three replicates.

In Study 1, data indicated that the gill actin gene might not contain an intron and was expressed in hemocytes. An actin-like gene, which might be an actin gene isoform without an intron, was amplified from gDNA. These results suggested that the partial actin gene sequence used in this study might not contain an intron. Although all sequenced vertebrate actin genes contain introns at amino acid residues 43, 270, and 330, the number and location of introns in invertebrate actin genes vary. For example, drosophila has 6 isoforms of actin genes. Isoforms 2 and 5 contain no intron (Fyrberg et al. 1981). Thus, for invertebrates, identification of the actin intron is needed before using an actin isoform as an internal control of RT-PCR. Meanwhile digestion of RNA with DNase I to eliminate the contaminated gDNA is required to avoid a false positive of actin RT-PCR when the actin gene or isoform actin genes contain no intron.

The tissue expression patterns of isoform actin genes (alpha, beta and gamma) are different in vertebrates and invertebrates. In vertebrates the alpha isoform actin genes are expressed only in muscle while beta cytoplasmic isoform actin genes are expressed in most tissues (Kabsch and Vandekerckhove 1992). Although the cytoplasmic and muscle actin of vertebrates can be distinguished by analyzing actin amino acid sequences, this is not possible in invertebrates (Vanderkerckhove and Weber 1978). Since the actin gene from gill was also expressed in hemocytes, the actin gene might be a cytoplasmic actin gene that is expressed in most tissues. Therefore, the gill actin gene can be used as a RT-PCR internal control for non-muscle tissues such as hemocytes.

Furthermore, the results of Study 2 is the first report on transferring of exogenous antimicrobial peptide genes into oysters to study their effect on oyster immunity. The results of the bacteria tissue loading assay indicated that treatments receiving

antimicrobial peptides genes had fewer bacteria than those of the control groups though no significant difference was found at $P < 0.05$ and $P < 0.2$ after log transformation of data.

PCR results from hemocyte DNA indicated that oyster hemocytes retained the transferred genes at least 3 days after transfection. However, RT-PCR results from hemocyte total RNA did not show detectable levels of cecropin B mRNA, despite the observations that bacteria tissue loading decreased in oyster groups transfected with antimicrobial peptide genes. The unstable cecropin B mRNA might be one possible explanation for the absence of cecropin B mRNA in hemocytes. The 3' end untranslated mRNA sequence (UUAUUUAU) present in the cecropin B gene and most known interlukins, tumor necrosis factor, human and mouse granulocyte-marcrophage colony stimulating factors (GM-CSF) can cause the rapid degradation of the mRNA (Kruys et al. 1994). RT-PCR on a series of samples taken at different times after transfection needs to be performed to determine the time course of cecropin B gene expression.

On the other hand, cecropin B and LOKI-24 might be expressed as functional peptides in other transfected tissues. For example, the cecropin B (APR) promoter was found to function not only in channel catfish hemocytes but also in fibroblast cells (Zhang et al. 1998). These results suggest that the APR promoter can function in other tissues. The decrease of bacteria tissue loading might be due to production of cecropin B and LOKI-24 peptides from other transfected tissues, which were not examined in this study. The effects of these peptides however, might be limited to extracellular bacteria.

The decrease in bacteria tissue loading was not likely caused by an increase in hemocyte number, since the hemocyte number of each group increased after transfection.

Despite the increase in hemocyte number in the gene transfer groups, the increase was about the same or smaller than that of the control groups (Table 5-2). Moreover, the hemocyte killing indices increased in saline transfected group oysters while other oyster groups showed a decrease of 40% to 55% in hemocyte killing (Table 5-2). Whether human pathogenic bacteria loading, i.e. *Vibrio* sp. decreased while total bacteria loading decreased, remains to be investigated by using a selection medium for *Vibrio.*

The gene transfer process affected oyster hemocyte numbers, the hemocyte killing index and bacteria hemolymph loading. An increase in hemocyte numbers in each group after transfection indicated that hemocytes in circulation increased. This might be due to the migration of tissue hemocytes to hemolymph (Oubella et al. 1993) or the proliferation of hemocytes. The 40% to 50% decrease of hemocyte killing ability in transfected oysters implied that the reduced activity of hemocytes (Figure 5-9) was due to the toxic effect of transfection reagent, which was observed in *in vitro* transfection assays. However, in time, the hemocyte killing ability might recover from the toxic effects of transfection. This could be demonstrated by a longer study with increased sampling points.

In addition to the decreased hemocyte killing index, plasma and proteases might play a role in increased bacteria hemolymph loading. The antimicrobial activity of PHOR-21, a synthetic antimicrobial peptide containing 21 amino acid residues, was found to be significantly decreased by oyster plasma (La Peyre et al. 1998a). The minimum inhibitory concentration (MIC) of PHOR-21 against *Vibrio vulnificus* increased from 1.56μM with no oyster plasma to 25 μM in the presence of oyster plasma. One of the major serum components found to inhibit antimicrobial activity was identified as a

low-density lipoprotein (Peck-Miller et al. 1993). In plants, the endogenous serine protease of tobacco leaf was shown to degrade cecropin B peptides (Florack et al. 1995). Moreover, exogenous proteases (virulence factors) from pathogens such as *P. marinus* could reduce the efficacy of PHOR-21 against *P. marinus* (La Peyre et al. 1998a). Since oysters used in this study were infected with *P. marinus*, oyster plasma and *P. marinus* proteases might have played a role in further diminishing the antimicrobial activities of cecropin B and LOKI-24 peptides.

The question remains as to why the bacterial tissue loading decreased but not the *P. marinus* loading in this study. The most probable explanation was that a higher concentration of lytic peptide was needed to kill or inhibit the growth of *Perkinsus*. The *in vitro* results of antimicrobial peptide toxicity to bacteria and protozoa support this explanation. For example, the MIC of PHOR-21 against *Vibrio vulnificus* was in the range of 1.56 to 3.12 μM while 50 μM was needed to kill 100% of *P. marinus* (La Peyre et al. 1998a). Moreover, the MIC of tachyplesin I, an antimicrobial peptide from blue crabs, for bacterial pathogenic *Vibrio* spp. (*Vibrio alginolyticus, V. anguillarum*, and *V. harveyi*), was between 0.4 and 1.6 μg/ml while 500 μg/ml of tachyplesin I was needed to kill 62% of *P. marinus* (Morvan et al. 1997). It was previously reported that 10 fold lower concentrations of antimicrobial peptide were required to kill bacteria as opposed to protozoan parasites (Jaynes et al. 1988). In this study, the peptide production might be limited and the concentration of antimicrobial peptide in transfected tissues was enough to decrease the bacteria load but not the *P. marinus* load. Improvement of transfection efficiency might increase the production of lytic peptides to a concentration to kill or

inhibit the growth of *P. marinus*. Or when toxic effects of transfection ceased (longer wait), there may be more cells to produce lytic peptides.

The highly variable physiological and immunological condition among individual oysters hindered detection of a significant difference among groups for the evaluated parameters. In this study, each parameter differed among oysters, regardless of the treatment. In evaluating eastern oyster hemocyte killing indices of bacteria, a large fluctuation was found even though 10 oysters were pooled per sample and contained 3 replicates of pooled samples (Genthner et al. 1999). The highly variable killing index for *Vibrio parahaemolyticus* was 16 ± 10% compared to *Listeria monocytogenes* strains, which was 18 ± 14% (Genthner et al. 1999). In the study with whole oyster *P. marinus* loading, variability was found regardless of how the oysters were infected, whether natural or experimental (Faisal et al. 1999). The mean of *P. marinus* tissue loading for wild oyster populations was $10.9 \pm 30.7 \times 10^6$ parasite/g tissue (n = 25, range = 1.53×10^8). Likewise, *P. marinus* free oysters which were then artificially infected with *P. marinus*, demonstrated highly variable parasite loading among individuals, with a mean of $3.2 \pm 4.7 \times 10^5$ parasites/g tissues (n = 15, range = 1.6×10^6). In a study of environmental and disease factors affecting mussel *Mytilus galloprovincialis* circulating hemocytes, a high level of variability was seen (Carballal et al. 1998). The mean hemocyte number was $3.3 \pm 0.10 \times 10^6$ hemocytes/ml (n = 540, range = 15.4×10^6). Due to the variability of the evaluated parameters within groups in our studies, detecting a significant difference among groups is difficult unless the treatment induced a large amount of change in the evaluated parameters.

To summarize our findings, oysters retained transferred genes in the hemocytes, and the transfection process led to an increase in circulating hemocytes but decreased the hemocyte killing ability for the duration of this study. Although the presence of cecropin B mRNA in hemocytes was not detected, the bacteria tissue loading decreased in oysters transfected with antimicrobial peptide genes. The results of this study are the first report to suggest the feasibility of enhancing oyster immunity and reducing bacteria loading with the transfer of antimicrobial peptide genes. Further studies should include the design of antimicrobial peptide genes for optimal expression in oysters. Factors leading to inadequate production of functional lytic peptides might be at the level of transcription, translation or post-translation modification of lytic peptide genes. Control of gene transcription by a promoter was discussed in Chapter 4. Translational and post-translational modifications are discussed below.

At the translation level, the use of genes from different animal species might stall translation due to rare codons used in the gene. For example, introduction of rare codons in yeast caused a dramatic decline of gene expression (Hoekema et al. 1987). Oyster codon usage (which can be obtained at the worldwide web site of the Kazusa DNA Research Institute, Japan, (http://www.kazusa.or.jp/codon/) is needed to design transgenes for oyster cells.

At the post-translational modification level, the pre-pro-cecropin B peptide might not function at an optimal level. There was a 22 amino acid pre-peptide sequence (signal sequence) followed by a 4 amino acid pro-peptide sequence at the amino terminus (N-terminus) of cecropin (Figure 5-1 B). The signal sequence is for directing the peptide to the endoplasmic reticulum (ER) for secretion. There, the signal sequence is cleaved by a

signal peptidase contained in the ER (Boman et al. 1989). The pro-sequence contains sub-cellular targeting information and keeps the antimicrobial peptide inactive before reaching its final destination, such as a phagosome or hemolymph (Valore et al. 1996). The pro-sequence is removed by dipeptidylaminopeptidase found at the target site for the antimicrobial peptide to activate it for full function (Kreil 1990). Although the pre-pro-sequence of cecropin B had been used to direct the secretion of cecropin and cecropin analogs in Sf9 insect cells (Choi 1996) and the secretion of cytosolic plant proteins in plant cells (Denecke et al. 1990), whether or not the pre-pro-sequence of cecropin B can be recognized by oysters needs to be determined for further improvement of the antimicrobial peptide secretion and sub-cellular targeting studies, or the identity of an oyster pre-pro-sequence must be determined.

Once the pre-pro-peptide was processed into a functional peptide, the antimicrobial activities could be affected by host factors such as pH, salt, and serum. These factors might decrease the activity of antimicrobial peptides and led to difficulty in detecting significant changes in the parameters being evaluated. The pH tolerance of cecropin B and LOKI-24 peptides require further evaluation. Antimicrobial peptides might be pH sensitive, for example at pH 5.5 to 8.0 no effect was seen on the MIC of PHOR-21 against *Vibrio vulnificus* (La Peyre et al. 1998b). Therefore, PHOR-21 could be expressed in the oyster hemolymph (pH 7.6) and still remain active (Boyd and Burnett 1996). On the other hand, clavanin, a pH sensitive lytic peptide from tunicate, *Styela clava*, hemocytes showed higher antimicrobial activity at pH 5.5 than at pH 7.4 (Lee et al. 1997). The low optimal pH of clavanin made it one of the best candidates for expression in oyster phagosomes, where the pH value is between 3.1 and 4.9 (Beaven and

Paynter 1999). The low pH in phagosomes can enhance clavanin activity while higher pH outside phagosomes or cells can reduce the clavanin toxicity to host cells (Lee et al. 1997). Therefore, reduction of *P. marinus* loading in oysters might be achieved more effectively using a lysosome oriented signal peptide sequence to direct pH insensitive antimicrobial peptides such as PHOR-21 or those that prefer low pH such as clavanin to lysosomes which fuse with phagosomes where *P. marinus* resides.

Finally, sensitivity of antimicrobial peptides to salt becomes an important factor to be considered due to the oyster's seawater environment. Although salt sensitivities of cecropin B and LOKI-24 were not evaluated, salt might affect their antimicrobial activity. The minimum concentration of PHOR-21 to inhibit the growth of *Vibrio vulnificus* increased 8 fold from 0.78 μM, at a salinity of 6 ‰, to 6.25 μM, at a salinity of 30 ‰ (La Peyre et al. 1998b). The antimicrobial activity of other peptides such as mouse and human beta-defensin-1 were reduced by salt (Tager et al. 1998, Bals et al. 1998). In contrast to salt sensitive PHOR-21 and beta-detensin-1, 5.9 to 17.2 ‰ NaCl concentrations did not significantly affect the antimicrobial activity of clavanins from tunicates that reside in seawater (Lee et al. 1997). The results of our studies implied that disease resistance of oysters could be enhanced, by transferring a properly designed lytic peptide gene.

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CHAPTER 6 SUMMARY AND CONCLUSIONS

The eastern oyster industry is economically important to the east coast of the United States. In addition to over fishing and the degradation of the habitat, diseases have caused a decline in oyster production. Traditional genetic improvements in oyster strains to enhance disease resistance have been conducted with limited success, while *in vitro* studies of disease are hampered by the lack of a well established cell culture system and cell line. Development of an optimal oyster cell culture system and cell lines have long been recognized as urgent and essential for disease studies. However, no systematic approaches have been conducted. Therefore, limited progress has been made during the past 40 years (Rinkevich 1999).

The goal of this work was to evaluate the feasibility of using antimicrobial peptide gene transfer to enhance oyster immunity. Included in this dissertation is the work that paves the road to reach that goal. First, a systematic approach was used to develop two basic needs for cell culture—tissue dissociation and cell cryopreservation. Second, based on the above results, an *in vitro,* serum-free gene transfer system was established. Third, the promoter of snail heat shock protein 70 gene and the promoter of insect cecropin B gene were evaluated using this gene transfer system. Fourth, antimicrobial genes controlled by the cecropin B promoter were transferred into juvenile oysters to evaluate their effects on oyster immunity.

Dissociation of tissue into individual cells is the first step to initiate cell culture, however, a systematic comparison of dissociation enzyme efficiency was not available. In the effort to address this, the dissociation efficiency of collagenase, pronase, and trypsin were compared in Chapter 2. Using cell number, cell viability, cell yield, and cell

dehydrogenase activity as parameters and repeating the experiment at least five times, pronase was found to be statistically the best among these enzymes to dissociate atrial, ventricle, and mantle tissues. This result indicated that enzymes such as collagenase and trypsin, usually used to dissociate vertebrate tissues, are not the best to dissociate oyster tissues. Meanwhile, this was the first quantitative comparison of enzyme dissociation efficiency and it set the foundation for further cell culture studies, such as the development of cell lines and *in vitro* gene transfer.

The conditions for cryopreservation of the dissociated atrial and ventricle cells were reported in Chapter 3. In order to preserve the dissociated cells for further studies, cryoprotectants were screened, and freezing rates and thawing temperatures were compared. The optimized cryopreservation conditions were 1) 10% glycerol, freezing at medium rate and thawing at 45° C for atrial cells, and 2) 10% glycerol, freezing at medium rate and thawing at 25° C for ventricle cells. These conditions are the first to be reported for preserved somatic oyster cells.

In addition to developing cell isolation and cell cryopreservation conditions, the establishment of an *in vitro* gene transfer system is required for studies of gene function such as gene regulation. In Chapter 4, the first serum-free gene transfer system was established. By using this system, the function of the snail heat shock 70 promoter (SHSP-70) and cecropin B promoter was evaluated. Optimal conditions to activate SHSP-70 by heat shock (stress) were developed, however, addition of LPS and IL-alpha did not appear to induce the cecropin B promoter. Further studies on the cecropin B promoter are needed.

In Chapter 5, the *in vivo* transfer of an antimicrobial peptide gene was conducted to evaluate the effect on oyster immunity. Although no significant difference was found among evaluated parameters, bacteria tissue loading of oysters receiving antimicrobial genes was lower than that of controls. These results imply that antimicrobial peptide gene transfer to enhance oyster diseases resistance is feasible.

The most significant achievement of this work was the establishment of a gene transfer system for oyster ventricle cells which are cultured in serum-free medium. Sera contain undefined components and the components of serum vary from lot to lot (Bjare 1992). Therefore, the results of studies using serum-free medium will be more reliable than those using a medium that contains serum (Bjare 1992). This gene transfer system will benefit further oyster studies including the screening of functional promoters, evaluating the function of potential disease resistance genes, identifying disease resistance genes from oysters, identifying virulence genes of oyster pathogens, studying the interaction between oyster cells and pathogens at the gene level, producing valuable peptides using oyster cells as bio-reactors, and monitoring environment pollution using oyster cells as bio-sensors.

By applying the knowledge of oyster cell *in vitro* studies, disease problems may be resolved by transferring antimicrobial peptide genes. Other quantitative phenotypes such as growth rate and quality phenotypes, such as nutrition value, may also be improved. However, the issues of food safety and ecological impact of transgenic oysters need to be carefully evaluated. The addition of extra genes into the oyster may alter other gene expressions and may lead to the change of protein compositions of oyster meats. Whether the change of protein composition is toxic or allergenic to humans must be

assessed. The potential negative impact of transgenic oysters on ecological system may be a problem because containment of cultured transgenic oysters is not possible compared to containment of transgenic animals in a land habitat. The evaluation of aquatic transgenic animal impact on an ecological system, such as cross breeding with other species is not possible. One of the strategies to minimize the impact on an ecological system is to produce sterile transgenic oysters by chromosome manipulation, radiation exposure, or deletion of the genes involved in gamete formation.

The acceptance of transgenic oysters by consumers should be emphasized, because the genetic improvement of animals by transgenic techniques developed during the last 20 years, is relatively new compared to the traditional breeding and selection technique developed thousands of years ago. Consumer awareness about transgenic techniques as well as environmental and food safety issues with transgenic animals, should be conducted in parallel with the development of transgenic animals as food.

In conclusion, the work of this dissertation developed an *in vitro* system that allows oyster studies to be conducted at the gene level. However, the hypothesis of adding an antimicrobial peptide gene to enhance oyster immunity cannot be validated, due to the high variation of the physiological condition of the oyster. Further *in vitro* and *in vivo* research is needed.

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APPENDIX A COMPOSITION OF DECONTAMINATION SOLUTION USED FOR TISSUE DECONTAMINATION

Antibiotic	Concentration (per/L)
Penicillin	500 units
Streptomycin	500 mg
Gentamycin	250 mg
Kanamycin	500 mg
Neomycin	250 mg
Chloramphenicol	25 mg
Polymyxin B	250 mg
Erythromycin	28 mg
Amphotericin B	28 mg

APPENDIX B COMPOSITION OF SALINE I AND SALINE II USED FOR DISSOCIATION SOLUTIONS

Ingredient	Saline I (g/L)	Saline II (g/L)
$CaCl_2$	0.480	-
$MgSO_4$	1.456	-
$MgCl_2 \cdot 6H_2O$	2.182	-
NaCl	11.611	16.600
KCl	0.310	0.595
$NaHCO_3$	0.350	0.666
Glucose	-	0.555
Galactose	-	0.111
Trehalose	-	0.111

APPENDIX C COMPOSITION OF JL-OPRD-4 MEDIUM

All chemicals were purchase from Sigma Chemical Co. St. Louis, Missouri unless otherwise indicated.

Table C-1. Preparation of 500 ml JL-ODRP-4 medium

Number	Solutions	Volume (ml)
1	Balanced salts solution*	454.0
2	Trace solution 1*	0.5
3	Trace solution 2*	0.5
4	Essential amino acid solution (Gibco BRL, Gaithersburg, Maryland)	5.0
5	Non-essential amino acid solution (Gibco BRL)	5.0
6	Amino acid solution*	5.0
7	Nucleotide / Coenzyme A solution*	1.0
8	RPMI 1640 vitamin solution	3.0
9	Carbohydrate solution*	5.0
10	Defined lipid solution (Gibco BRL)	5.0
11	Glutathione solution*	0.5
12	Aprotinin solution*	0.5
13	SPIT solution	5.0
14	Chloramphenicol*	5.0
15	Hepes solution	5.0

* Refer to Table C-2

Table C-2. Components of solutions for the JL-OPRD-4 preparation.

Solution	Components	Concentrations
Balanced salt/ buffer solution		
	$CaCl_2$	360.0 mg/L
	$MgSO_4$	1092.0 mg/L
	$MgCl_2 . 6H_2O$	1637.0 mg/L
	KCl	233.0 mg/L
	NaCl	8708.0 mg/L
	$NaHCO_3$	350.0 mg/L
	$Na_2HPO_4 . 7H_2O$	28.0 mg/L
Trace solution 1		
	Ferrous sulfate	83.4 mg/100 ml
	Zinc sulfate	14.3 mg/100 ml
Trace solution 2		
	Cupric sulfate	24.9 mg/100 ml
Amino acid solution		
	Alanine	1000.0 mg/100 ml
	Glycine	500.0 mg/100 ml
	Serine	500.0 mg/100 ml
	Taurine	1500.0 mg/100 ml
	Glutamine	500.0 mg/100 ml
Nucleotide/Coenzyme A solution		
	Adenosine 5'-Monophosphate	20.0 mg/40 ml
	Cytidine 5'-Monophosphate	20.0 mg/40 ml
	Uridine 5'-Triphosphate	20.0 mg/40 ml
	Coenzyme A	20.0 mg/40 ml
Carbohydrate solution		
	Glucose	5.0 g/100 ml
	Trehalose	1.0 g/100 ml
	Galactose	1.0 g/100 ml
Glutathione solution		
	Glutathione (reduced)	100.0 mg/100 ml
Aprotinin solution		
	Aprotinin	618.5 KU/100ml
Chloramphenicol		
	Chloramphenicol	50.0 mg/100 ml

APPENDIX D COMPOSITION OF LA-3 MEDIUM

All chemicals were purchase from Sigma Chemical Co. St. Louis, Missouri unless otherwise indicated.

Table D-1. Preparation of 500 ml LA-3 medium

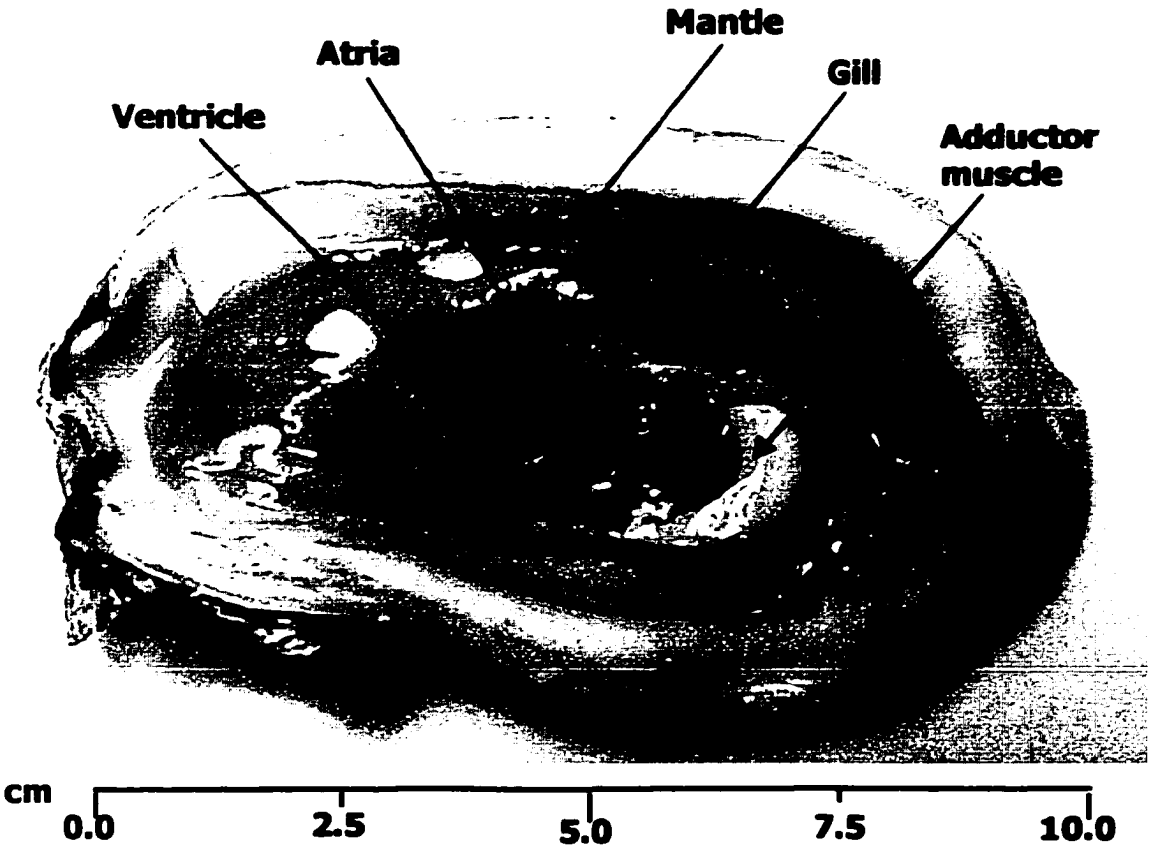
Number	Solutions	Volume (ml)
1	Balanced salts/buffer solution*	452.5
2	MEM essential amino acid solution	5.0
3	MEM non-essential amino acid solution	2.5
4	Carbohydrates solution*	5.0
5	X-tra amino acids solution*	2.5
6	X-tra glutamine solution*	2.5
7	RPMI 1640 vitamins solution	10.0
8	Antibiotics solutions*	3.0
9	Hepes solution	5.0
10	Soybean Trypsin inhibitor solution*	0.5
11	Mucin solution*	5.0
12	Nucleotides solution*	0.5
13	Vitamin E solution*	0.125
14	SPIT	1.25
15	Chemically defined lipid solution (Gibco BRL)	2.5
16	Linoleic acid-oleic acid-albumin	0.3125
17	Cholesterol (fresh)*	0.06125
18	Hydrocortisone*	1.25
19	Prostaglandin F-2α*	0.5

* refer to Table D-2.

Table D-2. Components of solutions for the preparation of LA-3 medium

Solution	Component	Concentration
Balanced salt buffer solution		
	$CaCl_2$ $2H_2O$	1029.0 mg/L dH_2O
	$MgSO_4$	1092.0 mg/L dH_2O
	$MgCl_2$. $6H_2O$	1637.0 mg/L dH_2O
	KCl	599.0 mg/L dH_2O
	NaCl	10051.7 mg/L dH_2O
	$NaHCO_3$	336.0 mg/L dH_2O
Carbohydrate solution		
	Glucose	5.0 g/100 ml
	Trehalose	1.0 g/100 ml
	Galactose	1.0 g/100 ml
X-tra amino acid solution		
	Alanine	1000.0 mg/100 ml
	Glycine	500.0 mg/100 ml
	Serine	500.0 mg/100 ml
	Taurine	1500.0 mg/100 ml
X-tra glutamine solution		
	Glutamine	2000.0 mg/100 ml
Antibiotic solutions		
	1.0 ml of Kanamycin stock solution	50.0 mg/ml
	2.5 ml of Gentamicin stock solution	10.0 mg/ml
	2.5 ml of Penicillin G stock solution	2 x 10^7 U/L
Soybean Trypsin inhibitor solution		
	Soybean trypsin inhibitor	100.0 mg/10ml
Mucin solution		
	Mucin	100.0 mg/100ml
Nucleotide solution		
	Adenosine 5'-Monophosphate	20.0 mg/40 ml
	Cytidine 5'-Monophosphate	20.0 mg/40 ml
	Uridine 5'-Triphosphate	20.0 mg/40 ml
Vitamin E solution		
	Vitamin E (α-tocopherol-acetate)	25.0 mg/ml ethanol
Cholesterol solution		
	Cholesterol	2.0 mg/ml ethanol
Hydrocortisone solution		
	Sterile Hydrocortisone	1.0 mg/ml ethanol
Prostaglandin F-2α		
	Sterile Prostaglandin	0.478 mg/ ml

APPENDIX E ANATOMY OF AN EASTERN OYSTER (*CRASSOSTREA VIRGINICA*)



APPENDIX F STANDARD OPERATION PROCEDURES

General cloning procedures were followed as described in the manual of Molecular Cloning (Sambrook et al., 1989).

Construction of Plasmid pAPR/GL3E

1. The plasmid pMON 200 containing the cecropin B genomic DNA was obtained from Dr. Jesse Jaynes, Department of Biochemistry, Louisiana State University.
2. Primers of CEP-1 with a *Kpn* I site (underlined) (5'-GAT-TAG-GTA-CCG-GGT-GTG-GTG-TAT-TCC-TGA-CCA-AAA-A-3') and CEP-2 with *Hind* III site (underlined) (5'-ACT-CTT-AAG-CTT-CGT-CGA-AAT-ATA-TTA-AAT-TTT-AAT-AAA-3') were designed using PC/Gene (Intellgenetics, Mount View, California) to amplify a 732 bp (from + 90 to – 641) fragment including 641 bp upstream and downstream 90 bp of transcription start site (+1) (Figure A-1).
3. The PCR products were digested with *Hind* III and *Kpn* I restriction enzymes (New England Biolab, Inc.), and electrophoresed in 1% agarose gel. The cecropin promoter was recovered using a gel extraction kit (Qiagene).
4. The amplified cecropin B promoter was cloned into a pBluscript KS cloning vector (Stratagene, La Jolla, California) for verification of the sequence.
5. The nucleotide sequence was verified using a Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Foster City, California), and an ABI Prism™ M 310 Genetic Analyzer (Perkin Elmer).
6. The verified cecropin B promoter sequence was released from the pBluescript KS cloning vector using *Hind* III and *Kpn* I restriction enzymes and cloned into *Hind* III and *Kpn* I multiple cloning sites of pGL3E containing a luciferase reporter gene.

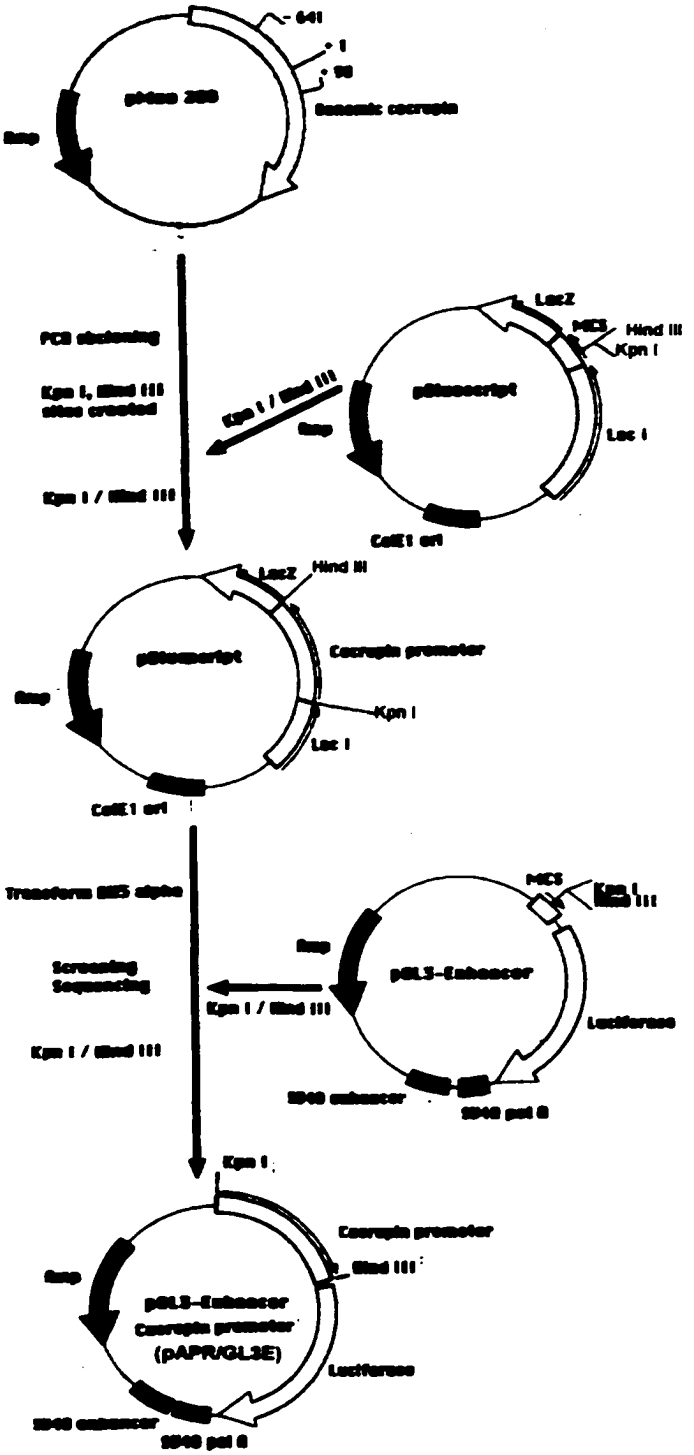


Figure A-1. Flow chart of construction of pAPR/GLE3

7. The pGL3E containing the cecropin B promoter sequence region was designated as pAPR/GL3E.

Construction of Plasmid pPC-6

1. The restriction enzyme *Bam* HI was used to remove a kanamycin-resistance gene from the Tn10 cassette of a modified mini-Tn10 transposon vector derived from pNK 2859 (Kleckner et al., 1991). Both sticky ends of the vector were filled to form blunt ends
2. Primers of FISH-1 (5'-CGT-GCT-AGA-GGG-GGT-TTA-ACT-TTA-ATG-TTC-3') and FISH-3 (5'-TCT-CCT-GAT-GTA-GTG-GCG-TGT-GGT-CGG-ATG-3') were designed using PC/Gene to amplify the 1886 bp fragment (from – 621 to +1265) of cecropin B genomic DNA from pMON 200.
3. The PCR products were sub-cloned into the vector above using blunt end ligation.
4. The nucleotide sequences were verified by sequencing.
5. The vector containing the correct sequences was designated as pPC-6.

References

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APPENDIX G UNANALYZED DATA

The raw data of this work was organized in the spread sheet of Microsoft Excel 2000, and saved in a CD containing the whole electronic version of this dissertation. The CD is available from Dr. Richard K. Cooper and Dr. Terrence R. Tiersch, Louisiana State University.

VITA

Ta Chih (Philip) Cheng was born in Taipei, Taiwan, Republic of China, on October 5, 1962. He received a master of science degree in biotechnology at Northwestern University in 1994. He then enrolled in the graduate program in the School of Forestry and Wildlife to study the aquatic animal genetics and then transferred to the Department of Veterinary Microbiology and Parasitology to study the aquatic animal diseases in 1995. He will received the degree of Doctor of Philosophy in August 2000.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Ta Chih Cheng

Major Field: Veterinary Medical Sciences

Title of Dissertation: Evaluation of Heterologous Promoter Function in the Eastern Oyster Crassostrea virginica

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

May 11, 2000